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INDUSTRIAL PHD PROJECT WITH NOVO NORDISK

BY
SIMON KAMENOV GAMMELGAARD

DISSERTATION SUBMITTED 2020

AALBORG UNIVERSITY
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Preface

This PhD thesis concludes the work by Simon Kamenov Gammelgaard in collaboration with Aalborg University and Novo Nordisk from 2017 to 2020. The industrial PhD project was funded by Innovation Fund Denmark under the Ministry of Higher Education and Science (grant nr. 7038-00004) and the Novo Nordisk STAR Fellowship Programme.

The thesis is constituted of three first-author manuscripts. Two are published and a third is accepted for publication at the time of submission of the thesis:

Manuscript 1 [1]

Characterization of Ultraviolet Photoreactions in Therapeutic Peptides by Femtosecond Laser Catalysis and Mass Spectrometry

> Simon K. Gammelgaard, Steffen B. Petersen, Kim F. Haselmann, and Peter Kresten Nielsen
> *ACS Omega* **2019** *4* (11), 14517-14525

Manuscript 2 [2]

Direct Ultraviolet Laser-Induced Reduction of Disulfide Bonds in Insulin and Vasopressin

> Simon K. Gammelgaard, Steffen B. Petersen, Kim F. Haselmann, and Peter Kresten Nielsen
> *ACS Omega* **2020** *5* (14), 7962-7968

Manuscript 3

Characterization of Insulin Dimers by Top-Down Mass Spectrometry

> Simon K. Gammelgaard, Steffen B. Petersen, Kim F. Haselmann, and Peter Kresten Nielsen

Journal of the American Society for Mass Spectrometry, special edition: MS in Industry. Accepted for publication 07OCT20.

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My department (*MS Characterisation*) in Novo Nordisk paved the way for me. I sometimes worry they made it too easy for me and spoiled me too much (not at all complaining though). My previous manager Christian Luber gave me the very important phone call that has led me all the way to here and made sure I had good start. Mette Dahl Andersen, who has been my manager for most of the time, has given me, especially in the hard last months where I was trying to finish off my PhD, an excellent support that I won't forget. All the technicians – Heidi, Janni, Gitte, Jan, Klaus, Lisbet, and Maggie – made my job significantly easier. Thanks to you I was able to efficiently conduct my experiments. The scientists have also been great colleagues.

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Steffen Petersen's photonics lab has a highly customised femtosecond laser setup. I would be surprised if any other protein research lab in the world can tap into the same possibilities as built by Steffen Petersen and his students. I

therefore benefitted highly from the technical efforts of the previous members of Petersen's lab who I never met except for Odete Goncalves. Odete was always positive, energetic, and very helpful with the photonics instrumentation.

There are so many in my personal life that if not for them I would not be here. Lise and Anders Gabel, who are no longer with us. There will be no one as good a human being as Anders. The remaining Gabel family. Birgit and my godfather Johs – I am truly sorry I have not spent more time with you the last many years. My bonus grandfather Tage, who recently passed away. Tage was so curious that I sometimes thought it was more suitable if we swapped so he was the scientist and I the cheesemaker. Ole, who came into my life at a critical time and was part of the solution. I am very thankful for my friends I got to know from elementary school as well as when studying biochemistry: Rasmus, Cathrine, Nicholas, Casper, Kine, Emil, Christian, Roall, Mie, and the remaining. Rose, who never complained whenever I came home later than expected or had to work in the weekends. When I broke the news that part of the project was in Aalborg she was a bit sad and worried but tried her best to hide it. My aunt and mother who deserves all the best in the world. To my little brother Aleksander: you are the best brother I could ever have been gifted.

Table of Contents

Abbreviations (English)

CW	-	continuous wave
DOPA	-	dihydroxyphenylalanine
ESI	-	electrospray ionisation
ETD	-	electron-transfer dissociation
EThcD	-	electron-transfer/higher-energy collision dissociation
GWG	-	Gly-Trp-Gly
GYG	-	Gly-Tyr-Gly
HMWP	-	high-molecular weight products
HCD	-	higher-energy collision-induced dissociation
laser	-	light amplification by stimulated emission of radiation
LC-MS	-	liquid chromatography-mass spectrometry
MS	-	mass spectrometry
MS^2	-	MS/MS or tandem MS
MS^3	-	MS/MS/MS
m/z	-	mass-to-charge
NFK	-	N-formylkynurenine
TOF	-	time-of-flight
UV	-	ultraviolet
UVPD	-	ultraviolet photodissociation

Summary (English)

Peptides and proteins are as amino acid polymers sensitive to ultraviolet (UV) light. The individual residues have intrinsic properties enabling absorption of energetic UV light that can destine the molecule into modification. The journey to the modification can be complex and challenging to predict for peptides and proteins. The chemical microenvironments created by folding can mitigate or promote the effects of UV light exposure. Tyr, Trp and the disulfide bond are the primary targets of modification at 280 nm. In the solution, UV light has been reported to induce reduction of disulfide bonds in a predominant model where UV excited Tyr or Trp donates the electrons. To study the UV photoreactivity of peptides/proteins in solution, continuous wave lamps are conventionally used to generate the photoproducts. Mass spectrometry (MS) is a suitable method to identify products and their individual modifications. A subdiscipline within MS, ultraviolet photodissociation (UVPD), uses UV lasers either at 193 nm or 213 nm to fragment the peptide backbone in the gas phase for analysis of the primary structure.

The shared activities across the manuscripts and unpublished results presented in this thesis is the combined utilisation of laser technology and MS to generate and describe UV photoproducts. The aims were to apply powerful femtosecond laser technology at 280 nm to rapidly accelerate and study the UV photoreactivity of peptides/proteins in the solution, and study UV photoproducts as agents carrying analytical value. A special interest was given to Tyr, Trp, and the disulfide bond. Five model molecules were selected: the tripeptides Gly-Tyr-Gly and Gly-Trp-Gly, the therapeutic peptides arginine vasopressin (one Tyr, one disulfide bond) and somatostatin-14 (one Trp, one disulfide bond), and human insulin (four Tyr, three disulfide bonds).

At 280 nm the femtosecond laser setup was able to accelerate photoreactions 40-fold compared to a continuous wave xenon lamp. MS analysis identified the same UV photoproducts between the pulsing and continuous source.

Within few minutes of femtosecond laser irradiation, UV photostability testing could be conducted and common as well as novel modifications were identifiable with MS analysis. The Tyr/Trp-mediated model of UV photo-induced reduction of disulfide bonds were studied. Interestingly, characterisation of the photoreduced products and 213 nm UVPD did not indicate or support any requirement for Tyr or Trp. We propose that UV photo-reduction in peptides/proteins can also derive from an additional model where the disulfide bond is cleaved by the direct absorption of UV light.

In human insulin the dominant UV reaction pathways led to high-molecular weight products (HMWP). The most abundant UV light-induced dimer was characterised directly with MS in a top-down approach. The type and sites of the crosslink was identifiable with the combined utilisation of diverse fragmentation methods. UVPD at 213 nm alone presented confident identification of the type and sites of the crosslink in a top-down analysis of another insulin dimer. Here, the UV light-induced fragmentation indicated that the chromophoric side chain of Phe participated in the crosslinking.

The following is concluded. Pulsing light sources of high intensity may replace lower-powered continuous sources for rapid UV irradiation of peptides and proteins in solution. UV light-induced reduction of disulfide bonds should not be exclusively interpreted as an outcome of Tyr or Trp absorbing the UV light but also as a probable outcome of the disulfide bond absorbing the UV light. State-of-the-art MS with diverse fragmentation methods is mature for top-down analysis of covalent insulin dimers. UVPD at 213 nm may have an extended application beyond backbone fragmentation in the characterisation of chromophoric crosslinks.

Resumé (Dansk)

Peptider og proteiner er som aminosyrepolymere følsomme overfor ultraviolet (UV) lys. De enkelte rester har iboende egenskaber der muliggør absorbering af energisk UV lys der kan afsende molekylet til modifikation. Rejsen til modifikationen kan være kompleks og udfordrende at forudsige for peptider og proteiner. Det kemiske mikromiljø skabt af foldning kan afbøde eller promovere effekterne af UV lyseksponering. Tyr, Trp og disulfidbindingen er de primære skydeskiver ved 280 nm. I opløsningen er UV lys blevet rapporteret til at inducere reduktion of disulfidbindinger i en udbredt model hvor UV-exciteret Tyr eller Trp donerer elektronerne. For at studere UV fotoreaktivitet af peptider/proteiner i opløsning, er kontinuertbølgede lamper konventionelt brugt til at generere fotoprodukterne. Massespektrometri (MS) er en egnet metode til at identificere produkter og deres individuelle modifikationer. En underdisciplin i MS, ultraviolet fotodissociation (UVFD), bruger UV lasere enten ved 193 nm eller 213 nm til at fragmentere peptidrygraden i gasfasen for analyse af den primære struktur.

Fællesaktiviteterne på tværs af manuskripterne og upublicerede resultater præsenteret i denne afhandling er den kombinerede anvendelse af laserteknologi og MS til at generere og beskrive UV fotoprodukter. Formålene var at anvende kraftfuld femtosekund laserteknologi ved 280 nm til at kraftigt accelerere og studere UV fotoreaktivitet af peptider/proteiner i opløsning, og studere UV fotoprodukter som agenter der indeholder analytisk værdi. En særlig interesse var givet til Tyr, Trp og disulfidbindingen. Fem modelmolekyler var udvalgt: tripeptiderne Gly-Tyr-Gly og Gly-Trp-Gly, de terapeutiske peptider arginin-vasopressin (en Tyr, en disulfidbinding) og somatostatin-14 (en Trp, en disulfidbinding), og human-insulin (fire Tyr, tre disulfidbindinger).

Ved 280 nm var femtosekundlaseren i stand til at accelererer fotoreaktioner 40 gange sammenlignet med en kontinuertbølget xenonlampe. MS analyse identificerede de samme UV fotoprodukter mellem den pulserende og

kontinuerlige kilde. Indenfor få minutter af femtosekund-laserbestrålingen kunne testning af UV fotostabilitet blive udført og almindelige såvel som nye modifikationer var identificerbare med MS analyse. Den Tyr/Trp-medierede model af UV fotoinduceret reduktion af disulfidbindinger var studeret. Interessant nok kunne karakteriseringen af de fotoreducerede produkter og 213 nm UVFD ikke indikere eller understøtte nogen krav for Tyr eller Trp. Vi forslår at UV fotoreduktion i peptider/proteiner også kan udspringe fra en yderligere model hvor disulfidbindingen bliver kløvet af den direkte absorbering af UV lys.

I human-insulin førte den dominerende UV reaktionsvej til højmolekylært-vægtede produkter Den mest udbredte UV lysinducerede dimer var karakteriseret direkte med MS i et øverst-ned tilgang. Typen og placeringen af krydsbindingen var identificerbare med den kombinerede anvendelse af diverse fragmentationsmetoder. UVFD ved 213 nm alene præsenterede overbevisende identifikation af typen og placeringen af krydsbinding i en øverst-ned analyse af en anden insulin-dimer. Her indikerede den UV lysinducerede fragmentation at den kromoforiske sidekæde af Phe deltog i krydsbindingen.

Det følgende er konkluderet. Pulserende lyskilder af høj intensitet kan muligvis erstatte svagere kontinuere kilder for meget hurtig UV bestråling af peptider og proteiner i opløsning. UV lysinduceret reduktion af disulfidbindinger bør ikke blive udelukkende fortolket som et udfald af at Tyr og Trp absorberer UV lyset men også som en sandsynlig udfald af at det er disulfidbindingen der absorberer UV lyset. Den nyeste MS med mangfoldige fragmentationsmetoder er moden for øverst-ned analyse af kovalente insulin-dimere. UVFD ved 213 nm kan muligvis have en udvidet applikation udover rygradfragmentering i karakterisering af kromoforiske krydsbindinger.

1 Background

The interaction between light and matter has many diverse essential impacts in our lives and in science. Life and atmosphere on Earth would not be as we know it today if it was not for the photochemistry of biomolecules. Sunlight energy is harvested in photosynthesis which fuels the foundation for most lifeforms and provide fundamental organic building blocks. In the reaction carbon dioxide is depleted from the atmosphere and molecular oxygen is released. In the eye, the same photoreceptor proteins follow the entire lifespan of the host to sense light. The absorption of light induces conformational change in the protein and is processed in the brain into vision. When gazing towards space, light can reveal elemental compositions of stars and planets. In peptide and protein chemistry, many important analytical applications use the interaction between light and molecule.

One type of interaction is absorption of the photon converted to electronic excitation. The energy of the photon must match the difference in energy between the ground state and an excited state of the chromophore in order to establish electronic excitation. The passage of energy barriers driven by the photonic energy can activate reaction pathways and have diverse roles in this thesis (**Figure 1**). In fluorescence, vibrational relaxation can be followed by spontaneous emission of a new photon, leading the chromophore to a safe return to its initial ground state without any covalent alterations. Emission from electronic relaxation is used in lasers to produce the intense light beams. The amino acid residues in peptides and proteins have electronic energy transitions that match the energy of UV light. Besides fluorescence (and phosphorescence), other pathways may result in fragmentation of covalent bonds, crosslinking, electron release, and transfer of the excitation or electron to other groups that are then modified or induce modification elsewhere. Characterisation by MS of these products provide knowledge of the photoreactivity and can provide analytical value of the parent molecule. In the last two decades the synergy between UV lasers and MS for structural analysis of fragmented biomolecules in the gas phase has increasingly improved in the

discipline known as UVPD. Other researchers have an interest in the solubilized UV photoproducts of peptides and proteins from a perspective of stability using lamps as light sources. Here, liquid chromatography-mass spectrometry (LC-MS) is a natural choice for characterising the chemical composition of the UV photoproducts.

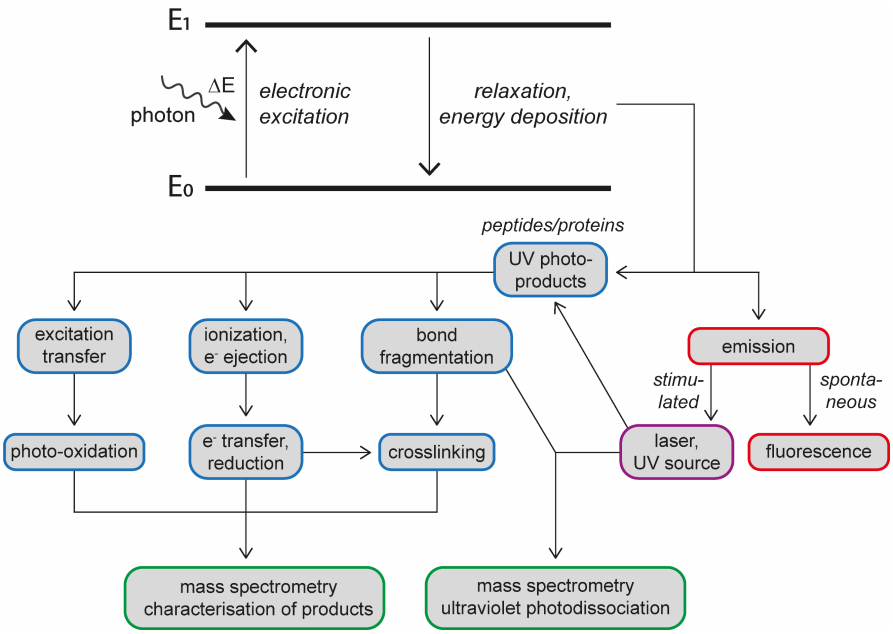

Figure 1. How keywords, reactions, and methods in the thesis are correlated to the effects of light-induced electronic excitation.

1.1 The UV photochemistry of peptides and proteins

The spectroscopic characteristics of peptides and proteins have diverse implications in protein chemistry, depending on the context. For biopharmaceuticals any degradation throughout the manufacturing and storage must be kept to a minimum for product quality and patient safety. Light-stressed biopharmaceuticals have the potential to pose a risk for the patient, as demonstrated in an animal study with light-induced aggregates of IgG1, which induced a strong immunogenic response in mice [3]. A guideline to forced degradation by UV light is available to decide whether the

biopharmaceutic products should be protected from light with packaging [4]. The UV spectroscopic properties of peptides and proteins can be utilized for concentration determination, interaction and folding studies, detection in chromatography, and analysis of the primary structure by MS. A typical UV spectrum of a peptide or protein will exhibit two local absorption maxima from ~190 to ~230 nm and at ~280 nm [5-10]. Trp, Tyr, and the disulfide bond contribute significantly to both wavelength regions, while the peptide bond, Phe, His, Pro, and Met absorb in the ~190-230 nm region. **Figure 2** provides an overview of selected chromophores in the polypeptide and their extinction coefficients at 214 and 280 nm.

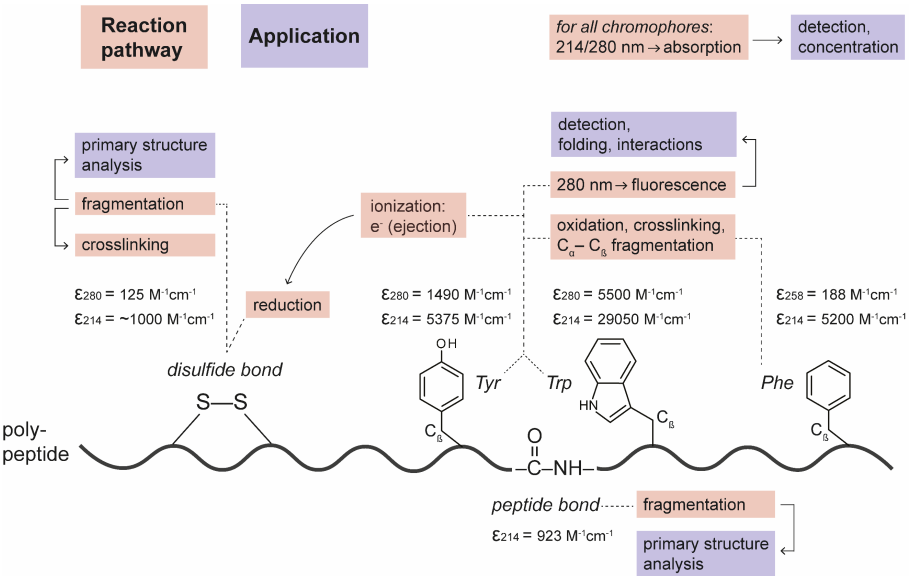

Figure 2. Overview of selected UV chromophores in a peptide important in analytics and vulnerable to UV photo-induced alterations. The remaining significant chromophores not depicted are His (ε_{214} = 5125 $M^{-1}\,cm^{-1}$), Pro (ε_{214} = 2675 $M^{-1}\,cm^{-1}$), and Met (ε_{214} = 980 $M^{-1}\,cm^{-1}$).

Based on the primary structure an expected absorptivity for UV photons can be deduced merely by summarizing the extinction coefficients of all the chromophores at the wavelength of interest. Beyond the individual chromophores in peptides and proteins, the UV photoreactivity is additionally

impacted by the structure, which defines spatial proximities between the residues and which residues interact with external chemical groups. These local chemical environments may shield or promote covalent modification, depending highly on the specific molecule. Especially exposure to solvent, crosslinking, hydrogen abstraction, and electron transfer between residues are heavily dictated by structure. UV photoreactions that may depend more on the isolated intrinsic properties of the chromophores are ionization and fragmentation of the covalent bonds. But even here the formed radicals can in secondary reactions be influenced by structure.

Solvent-exposed Tyr and Trp residues can be vulnerable to UV photo-oxidation [11, 12]. The UV excitation of the aromatic side chain can be transferred to molecular oxygen. The resulting singlet oxygen is reactive and may modify the same aromatic groups or due to diffusion in the solution modify residues elsewhere in the protein susceptible to oxidation. Among the most common photo-oxidation product of Tyr is dihydroxyphenylalanine (DOPA) and of Trp it is kynurenine by the addition of one oxygen or N-formylkynurenine (NFK) by the addition of two oxygen [13-18]. Oxidation is one of the major consequences of light exposure, as exemplified by the frequent reporting of photo-oxidation products of monoclonal antibodies [19-22]. While photo-oxidation can be induced by the surroundings, there is a risk it also appears in UV spectroscopy studies as unwanted artefacts that disturb the analysis. A recent study demonstrated with an LC-UV-MS setup that peptides, proteins and other biomolecules can be significantly oxidized when using high-performance UV detection [23].

A direct damage on the aromatic side chains of Tyr and Trp upon UV electronic excitation is ionization when an energized electron is ejected. The cationic tyrosyl ($Tyr^{\bullet+}$) or indolyl radical ($Trp^{\bullet+}$) can rapidly deprotonate into the neutral radical variant. The neutral tyrosyl ($Tyr^{\bullet}$) or indolyl radical ($Trp^{\bullet+}$) may pass on the radicalisation elsewhere by hydrogen abstraction or directly induce crosslinking to other aromatic groups nearby. Di-tyrosine formation has been suggested as a direct consequence of UV light exposure [24-26]. In a recent UV photostudy of α-lactalbumin, di-Tyr, di-Trp, and Tyr-Trp

crosslinks were all identified [27]. Solution studies of peptides and proteins have reported UV photoproducts that were fragmented within the side chain of Tyr and Trp at the C_α-C_β bond [28-31]. It was proposed that ionization into tyrosyl or indolyl radicals induce fragmentation of the C_α-C_β bond by passing the radical to the backbone C_α atom. UV photochemistry studies in the gas phase also report that fragmentation is possible at the C_α-C_β bond of Phe, Tyr, and Trp [32-34].

The thiyl radical (-S$^\bullet$) is another important intermediate in the UV photochemistry of peptides and protein. The cleavage of the disulfide bond upon direct absorption of the UV photon or one-electron reduction from UV light-induced ionization of Tyr or Trp yield thiyl radicals that may cause hydrogen abstraction and thioether crosslinking [35]. A commonly reported UV photomodification of the disulfide bond derived from sulfur radicalization is dithiohemiacetal (R_1-CH(-SH)-S-R_2). The dithiohemiacetal group has the irreversible thioether crosslink and a thiol that can participate in reversible reactions. The Schöneich lab has reported the formation of dithiohemiacetal by UV irradiation in a model peptide of 12 residues, the biological nonapeptide oxytocin, and the proteins IgG1 and human growth hormone [36-39]. Free thiols are vulnerable to re-oxidation forming scrambled disulfide bonds, however scrambled dithiohemiacetal products have not been reported and discussed in the literature before this work. Scrambling of disulfide bonds as a consequence of UV light exposure has been observed in lysozyme, α-lactalbumin, and IgG1 to cause aggregation [22, 27, 40, 41]. The mechanisms behind the disruption of the disulfide bond by UV light are studied both in the solution and gas phase, and are further discussed in *chapter 1.3*.

The peptide bond, His, Pro, and Met are generally not perceived as chromophores directly vulnerable to UV light exposure in UV photostability studies of peptides/proteins in solution. They have none or negligible absorption above ~230 nm where photostability begins to be relevant. However, in tandem MS analysis of which the analytes are in the gas phase, the UV absorption capacity of the peptide bond can be used to selectively fragment the backbone with lasers at 193 or 213 nm. In the gas phase

photochemistry field, fragmentation of the covalent bond by UV light is termed UVPD and described in more detail in *chapter 1.2.3*.

1.2 Characterisation of biotherapeutics by mass spectrometry

Proof of compound identity, assessment of purity as well as stability are required in the research and development of a peptide or protein as a biopharmaceutical candidate. The description of the biopharmaceutical down to the residual level is required to have complete confidence that the expected compound have been expressed and purified. The identity of the compound may be divided into two parts. The first is the primary structure as dictated by the expression vector. The second is the diverse modifications, originating from the post-translational machinery in the expression host cell or from rational design by the synthesis scientist, which modulate the behaviour of the molecule. After the biopharmaceutical has been expressed, modified, and purified as wanted, external factors such as formulation, heat, and light may induce degradation *in vitro*. The derived masses from MS can be interpreted into a feasible chemical composition and identity that is utilized to characterise some molecular features of biopharmaceuticals and their degradation products, such as the sequence or the modifications and their residual sites.

1.2.1 The disulfide bond

A common post-translational modification is the disulfide bond. Disulfide bonds have a key role in the structure and function of peptides and proteins. Multiple disulfide bonds can exist on the same molecule in complex patterns. A disulfide map is a description of which cysteine residues in the molecule are bridged together. Having obtained maps of disulfide bonds in biopharmaceuticals enable scientists to evaluate if the structure and ultimately the biological activity have been preserved throughout the biochemical conditions in the host cell and throughout the *in vitro* conditions. Identification of the disulfide connectivities in a protein can be achieved with the three-

dimensional structure methods nuclear magnetic resonance spectroscopy, X-ray crystallography, and more recently also state-of-the-art cryogenic electron microscopy. However, in an industrial setting with narrow time frames, the speed of and resource spend on the applied methods are important. The three-dimensional methods are all resource-heavy and comprehensive, generally causing them to be applied accordingly if there are no other methods that can answer the scientific questions.

MS is the most suitable method for initial attempts to map disulfide bonds in peptides and proteins [42]. Protease digestion is used to separate the Cys residues on the primary structure. The extend of successful proteolytic separation of Cys residues dictates the difficulty and efforts in mapping the patterns. In the optimal scenario all Cys residues are separated on the primary structure. These produced species contain the identity of the disulfide bonds in simple systems of two digested peptides connected through one disulfide bond. An additional MS analysis of the reduced sample can support the identifications by the depletion of the assumed disulfide-bonded systems. If the Cys residues are closely-spaced with no protease site for digestion, the disulfide-bonded species generated from the digestion can contain complex networks of multiple disulfide bonds between two or more digested peptides. Here, a key principal in the MS mapping procedures is that the reducing agent must generate a population of heterogeneously partially reduced species each with one remaining disulfide bond that contain the information needed for assigning the Cys pairs. Conventionally, reduction is conducted with chemicals like tris(2-carboxyethyl)phosphine (TCEP) or dithiothreitol [43]. However, there can be various challenges associated with chemical reduction, such as laborious sample preparation and unsuccessful production of specific partial reduced species. At the same time many next-generation larger peptides and proteins in novel therapeutic areas have complex disulfide bond patterns that pose an increasing analytical challenge for efficient MS analysis. Novel approaches to reduce disulfide bonds online to LC-MS analysis have therefor been of interest and developed, such as electrochemical reduction or in-source reduction [44-48].

1.2.2 Liquid chromatography-mass spectrometry (LC-MS)

The fundamental principal behind MS is the use of electrical or magnetic fields to manipulate the movement of analytes to acquire mass values that reflect the identity of the analytes. There are many diverse technological solutions to MS. What all mass spectrometers have in common in instrumentation is a dedicated ionisation source, a mass analyser, and a detector. In the ionisation source the analytes are brought to the gas phase charged, a prerequisite to control the motion of the ions in the MS instrument. The analyte ions are transported from the ionization source to the mass analyser which separates the ions in time or space for subsequent detection and processing into mass-to-charge (*m/z*) ratios that are further calculated to masses.

Peptides and proteins have since the invention of MS in the early 20th century been very challenging to analyse until the late '80s or early '90s. While small hydrophobic molecules were analysable by MS through evaporation and hard ionization, the polar and larger peptides/proteins were not compatible with the same techniques used for the smaller and more robust molecules [49]. The challenges with peptide/protein analysis were to bring the molecules to the gas phase and preserve the entire covalent structure. If it succeeded to bring peptides to the gas phase through heating, they fragmented due to their polar (and thereby covalently weak) bonds in the backbone or by the following hard ionization, losing the intact molecular ion. MS analysis of peptides and proteins were revolutionized by two soft ionization techniques that solved both challenges: electrospray ionisation (ESI) and matrix-assisted laser desorption/ionization (MALDI).

ESI is applied to dissolved samples that can add charge to the analytes and vaporize the water molecules surrounding the analyte ions. An electric field is applied to the solution as it passes through a capillary. A positive polarity is used for peptides and proteins to ensure an excess of protons used for the charging of the analytes, further facilitated by acidic conditions. Surface tension and electrostatic repulsion at the capillary tip cause the sample to produce an aerosol of continuously smaller droplets, additionally facilitated

by desolvation. As the droplets dry and charge is accumulated, the analytes are increasingly protonated and eventually depleted from interaction with water. The release to gas phase is primarily described by two mechanisms in which the ion is ejected out of the droplet or the droplet is completely evaporated leaving behind the ion [50]. The major strength and reason for why ESI is the most-used soft ionization technique for MS analysis of biomolecules is its direct compatibility and synergy with liquid reverse-phase chromatography. Before ESI, analytical chromatography of peptides and proteins mainly applied UV absorption for the detection. ESI enables the powerful hybridization of LC-UV with MS for an online coupling between separation, UV detection, and MS detection. Having reversed-phase LC as the inlet for MS analysis enables desalting for improved MS detection. Even more importantly, MS analysis of complex samples is heavily improved with LC separation and in many cases an essential requirement. In LC-UV-MS analysis of a sample with multiple analytes, the ideal scenario is a sequential and defined elution of each analyte species. As all the analyte species are not introduced to the MS inlet simultaneously, sensitivity of each analyte species is increased because competition is avoided for ESI and the MS spectra are decreased in complexity. The UV detection is placed between the outlet of the chromatographic column and the MS inlet. For the same analytes complementary UV- and MS-detected chromatograms are collected. The MS intensity is heavily depending on the number of basic residues that are protonated, which are sequence-specific. UV detection is independent of ionisation efficiency, and usually preferred over MS detection for quantification. UV absorption spectra of the individually-eluting analytes can also be collected online to the LC. Also, an inherent weakness of MS is characterisation of any chemical alteration not followed with a shift in mass. LC may here salvage MS if the isomeric species are separable. To ensure the best MS analysis of biopharmaceuticals, their modified variants, and degradation products, time-of-flight (TOF) or Orbitrap technologies are usually preferred over low-resolution mass analysers mostly covered by quadrupoles and ion traps. MS analysis of macromolecules such as larger

peptides and proteins benefit significantly from the accuracy and resolution provided by TOF and Orbitrap mass analysers. With sufficient resolution, ions of similar masses present in the same MS acquisition event can be distinguished by the generation of the individual *m/z* signals. At some hypothesized lower resolution these ions would contribute to the same *m/z* signal. Resolving closely-spaced *m/z* signals reveal the presence of multiple different analytes or within the individual analyte species distinguish the isotopic subpopulations for more accurate and confident identification.

1.2.3 Tandem mass spectrometry and UVPD

TOF and Orbitrap instruments are usually hybridized with a quadrupole, which on its own can function as a low-resolution scanning mass analyser but in a hybridized setup has another purpose. Here, the quadrupole is situated before the high resolution mass analyser and functions as a mass filter that only allows ions within a specified *m/z* window to safely translocate further in the instrument. In this way one species of interest, in a simultaneous introduction of multiple analyte ions of varying *m/z* values, can be isolated and exclusively selected for further MS events. These hybrid instruments with two or more different mass analysers in tandem enable tandem MS experiments. The biggest strength and what is utilized the most in tandem MS is the ability to fragment isolated ions for primary structure analysis. Tandem MS is usually abbreviated MS/MS or MS^2. However, certain MS instruments with ion trapping capabilities are not limited to one fragmentation step before the MS^2 scan. They are able to isolate the fragment (otherwise included in a MS^2) scan and further dissociate for a MS^3 scan or again continue the dissociation for MS^n analyses. The fragmentation of ions in the mass spectrometer can be activated by various approaches such as collision, electrons, or light. Probably two of the currently most-used and one intensively studied activation methods are, respectively, higher-energy collision-induced dissociation (HCD), electron-transfer dissociation (ETD), and UVPD. The ion population of the isolated precursor are distributed to dissociative pathways upon activation. The resulting product ions are divided

in subpopulations that share fragmentation of the same covalent bond and thus collectively contribute to the same *m/z* signal. Elucidation of the covalent structure of the precursor becomes possible when the *m/z* values, representing particular fragmentation sites on the molecule, are interpreted combinedly. The peptide backbone within each residue has three covalent bonds that can be fragmented. Dissociation of a backbone bond will yield a C- and N-terminal fragment pair. Only fragments carrying charge are detected and therefore both termini fragments of a pair are not always observed. A nomenclature by P. Roepstorff and J. Fohlman, *1984*, has been established of the product ions that describe which backbone cleavage in which residue in the sequence the fragments originate from (**Figure 3**) [51]. Collisional

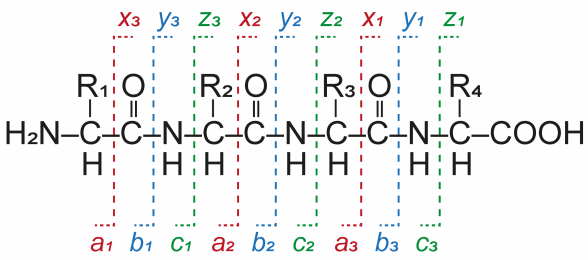

Figure 3. Nomenclature of the product ions derived from fragmentation in tandem MS, here illustrated on a tetrapeptide.

activation like HCD usually yield *b/y*-ions and electrons like in ETD usually yield *c/z*-ions. UV photons in 193 or 213 nm UVPD can yield all the aforementioned as well as *a/x*-ions. Hybridized activation methods such as the combination of ETD and HCD (EThcD) are also applied to produce spectra rich of *b-, c-, y-,* and *z*-ions.

UVPD is still a relatively exclusive activation method only commercially supported at 213 nm since 2017 as an integrated solution by Thermo Scientific (who are also the sole providers of the Orbitrap technology). Leading academia groups in the field have mostly reported UVPD at 193 nm [52, 53]. The ArF excimer lasers applied for the 193 nm irradiation are more powerful and match better the ~190 nm maximum absorption of the peptide bond than the solid state Nd:YAG laser for the commercial 213 nm UVPD. Irrespective

of the UV laser, the energy from an absorbed UV photon induce electronic excitation that can activate two overall pathways leading to dissociation of covalent bonds. The energy of the electronic excitation may be deposited by relaxation through vibration or deposited to direct dissociation of a covalent bond. In a vibrational relaxation the electron reaches the ground state by distributing the energy internally in the molecule. In tandem MS a vibrationally hot peptide or protein can channel the excitation into dissociation of the weakest bonds, which usually is the peptide bond. Collisional activation methods such as HCD cause vibrational excitation and therefore also generate the *b/y*-ions (which are products of the peptide bond fragmentation). In a direct dissociation upon electronic excitation the energy is immediately driving fragmentation and not internally converted in the molecule to distributed vibration. Electronic excitation can thereby fuel higher-energy dissociation pathways not available through vibrational excitation. In direct dissociation little or no energy remains for vibration. In ETD the electrons inducing dissociation have low energy and therefore do also not cause significant vibration. Larger peptides and proteins for the most have some structure in the gas phase held by non-covalent interactions. Vibrations can disrupt the low-energy non-covalent interactions. For those reasons is it common in ETD to observe reduced charge states of the precursor which contain fragmentation. However, the product ion pairs are still binding together non-covalently, leaving no analytical information of the primary structure. In EThcD, heating of the reduced precursors in the HCD step after ETD not only contributes to the observation of the *c/z*-ions by their non-covalent dissociation but can also be a strict requirement to observe ETD fragments [54, 55]. Since all fragment ions of the peptide backbone can be observed for one activation step it appears in UVPD that a unique synergy exists between direct dissociation and internal conversion. The vibration supports MS detection of directly-dissociated fragments and additionally enrich the spectra with its own fragments [56]. This synergy may also be the reason why UVPD can be applied successfully on intact proteins. New discoveries of UVPD are still reported that expand our understanding of the UV photodynamics of peptides/proteins. A recent

work has reported that aromatic residues can function as additional chromophores to increase the 213 nm UVPD of the backbone regions they are localized to [57].

1.2.4 Top-down mass spectrometry

The analysis of the intact protein as it enters the mass spectrometer and then deconstructed to smaller pieces by activated fragmentation is termed top-down MS. In the characterisation of the primary structure in larger peptides or proteins, the gas phase fragmentation in top-down MS could ideally replace the in-solution protease digestion used in bottom-up MS. The continuous improvements in software and MS technology such as faster scan rates, higher sensitivity and resolution, as well as in diversity of activation methods within the same instrument, all make top-down MS an increasingly attractive alternative or complement to bottom-up MS workflows. An instrument capable of powerful high resolution top-down analysis is the Orbitrap Fusion Tribrid [58]. HCD, ETD, and 213 nm UVPD are available for any MS^n step to combine as wanted in fragmentation workflows.

While bottom-up MS leads to significant and important lower technological requirements to mass spectrometers, inherit disadvantages do follow. Due to the nature of protease digestion, any information describing connections between post-translational modifications is lost when the modifications are divided to digested peptides. The modifications in the proteoforms can be studied in concert in top-down MS [59]. Other disadvantages of bottom-up workflows for characterisation of larger peptides and proteins, that are diminished or avoided in top-down workflows, are time-consuming and laborious sample preparation, incomplete protease digestion, loss of digested peptides e.g. from precipitation, and long LC gradients for the peptide mapping. As the sequences lose their native structure in the intact protein when digested and released to the solution as individual peptides, solubility can be decreased and stabilisation towards chemical degradation (such as oxidation and deamidation) may be weakened. In worst case degradation

artefacts are introduced when assessing the chemical stability of peptides and proteins.

Top-down MS has other challenges. Demands for state-of-the-art instrumentation and diversity in activation methods are higher than for bottom-up. As the backbone increases in length, the precursor ions and the charges are correspondingly diluted in the fragmentation in an unequal distribution throughout the sequence, challenging the lower detection limit of the mass analyser. The fragmentation spectra increase in complexity with size and may require software analysis such as ProSight Lite [60] for efficient and successful interpretation of the data but with the risk of false annotations. Disulfide bonds introduce an additional covalent bond that has to be fragmented in order to observe product ions in the sequence flanked by the two Cys residues. The requirement of simultaneous fragmentation of the disulfide bond and the peptide backbone translates in the best case to a diminished but still detectable abundance of the product ions and in the worst case causes a complete darkening of the sequence coverage. ETD, and UVPD in one study, have proven more efficient than HCD in cleaving disulfide bonds in peptides or proteins and may provide product ions not observable with HCD [61-67]. In the following chapter UVPD of the disulfide bond will be covered

A common benchmarking of the progressions in the field of top-down MS is sequence coverage in model proteins. State-of-the-art top-down MS and software analysis has reported ~40% sequence coverage on a 150 kDa IgG1 [68]. Before this work only three studies have reported top-down characterisation of crosslinks in proteins, likely due same challenges in fragmentation as for disulfide bonds [69-71].

1.3 UV light-induced disruption of disulfide bonds in peptides/proteins

The majority of this subchapter has also been used in an introduction for a PhD plan report to the doctoral school. Some sentences are different.

The UV photo-induced cleavage of disulfides have been studied both in the context of gas phase [52, 67, 72-74] and in solution [26, 36, 38, 40, 41, 75-

82]. The research of disulfide UV photoreactions can therefore also be divided into one of two fields, depending on the physical phase. Generally, fragmentation of disulfides in the gas phase is studied in the context of analytical peptide/protein chemistry. Usually the UVPD experiments are conducted in an ion trap in a mass spectrometer installed with a laser. UV photo-induced cleavages of disulfides in the solution are mainly identified and discussed in the context of characterising the photostability of peptides/proteins. The usual workflow is continuous irradiation of the disulfide-containing molecule followed by characterisation with spectroscopy, MS, or sometimes both. In the two fields there are roughly two different views on how the disulfides are cleaved based on which chromophores absorb the photons.

In gas phase studies, disulfides are explained to be cleaved through directly targeting the disulfide with UV photons. The maximum absorption of the disulfide bond in cystine is around 150 nm and 157 nm laser irradiation was therefore used to fragment the bond in the gas phase [72]. Proof-of-concept disulfide photodissociation was observed but the high energy photons also resulted in peptide backbone fragmentation. Higher wavelength was later on explored in the field. It was shown that laser irradiation at 266 nm can selectively cleave disulfide bonds of a disulfide-linked peptide pair while preserving the peptide backbone [73]. A study later on explored the fragmentation mechanisms of a disulfide bond in free cystine at 263 nm, proposing that the disulfide upon absorption of a UV photon causes the homolytic dissociation of the two sulfur atoms into two thiyl radicals [74]. Others have demonstrated that 193 nm UVPD is capable of fragmenting the two intermolecular disulfide bonds in insulin, releasing its A and B chain in the mass spectrometer [67].

In solution phase studies, the UV light-induced reduction of disulfide bonds are dominantly explained by a different mechanism involving Tyr/Trp-mediation. The Petersen lab has previously reported that Trp in the disulfide-containing enzyme cutinase can eject electrons into the solution upon excitation of 295 nm photons [79]. They suggested that the solvated electrons

can reduce disulfide bonds. Two years earlier another study of cutinase reported observations of a lost disulfide bond upon UV irradiation and also proposed that Trp was the electron source [75]. It appears the two cutinase studies may have been inspired by previous findings of which the UV photoreduction of the disulfide bond in free cystine is promoted by the presence of free Tyr or Trp [83-85]. This model based on aromatic residue mediation was subsequently used by researchers from two separate groups to explain their observations on UV-irradiated α-lactalbumin [76-78]. Both groups detected the generation of free thiols and discussed the three-dimensional relationships and distances between the Trp residues and disulfides to rationalize Trp-mediated reduction. Another example of a group adopting Tyr/Trp-mediated UV photoreduction with limited experiments to support the model is based on the scrambled intermolecular disulfide bonds of lysozyme [40, 41]. In 254 nm irradiation experiments of human insulin, an electron scavenger was used to collect the solvated electrons from excited Tyr residues [81]. The data supported and accounted for 33% percent of the generated thiols to be dependent on solvated electrons. They further proposed that the remaining 67% of the thiols in insulin are generated by a direct absorption of the photon by the disulfide leading to the dissociation of the two sulfur atoms, which is also the explanation model used in gas-phase UVPD. However, with a 254 nm local maximum a decreased in Tyr/Trp-mediation is expected compared to 280 nm irradiation.

Overall it appears that these two subfields of UV photochemistry each have their predominant model for the UV light-induced cleavage of the disulfide bond. Also, the referencing and discussion across the fields in the literature is rather limited.

A third methodology to cleave disulfide bonds with UV light is researched by the Yu Xia lab. In 2017 the interaction between UV light, methanol, and disulfide bonds in peptides and proteins were demonstrated to produce stable cleaved products of disulfide bonds [86]. One sulfur is reduced into a thiol and the other is capped by the methanol into a sulfenic methyl ester (-$SOCH_3$). Among the molecules included in the study was bovine insulin of which one

of the interchain disulfide bonds was identified by tandem MS to have been cleaved. In a subsequent work the efficiency of the disulfide bond cleavage appears to have been improved by including acetone to the combination of UV light and alcohol [87]. Furthermore, the ratio between the capped sulfur and thiol was illustrated to be changeable by methanol, ethanol and isopropanol. UV irradiation was conducted in a microflow reactor with a flow of 1-10 μL/min before the infusion. An efficiency of the simultaneous reduction of the interchain disulfide bonds in porcine insulin was 88% after just 3 s of UV irradiation with the acetone/isopropanol mixture. Extensive UV irradiation may have been achieved due to the low sample volume in the μL range in a capillary ensuring efficient exposure to the UV light.

1.4 Lasers

Laser is an abbreviation for "*l*ight *a*mplification by *s*timulated *e*mission of *r*adiation". Any light source using stimulated emission to produce the output is a laser. The emission of light derives from the relaxation of an excited electron in an atom, ion, or molecule to the ground state. Normally when a photon is absorbed and converted to an electronic excitation, the emission of a new photon is a spontaneous process dictated by the life-time of the excitation, known as fluorescence and phosphorescence. Spontaneous emission produces photons in random directions. The emission can also be prematurely activated by the passage of a nearby photon with energy matching the transition from the excited electronic state to the ground state. The new photon is identical to the characteristics of the passing photon. The wavelength, phase, and direction of the passing photon are thereby amplified by the stimulated photon. This coherency of the light beam is a unique feature of lasers. The stimulated emission and amplification takes place in a material called the medium. Energy from an external source must be pumped to the medium for excitation to occur in the atom, ion, or molecule. The initial excitations by the pumping relax spontaneously to photons in random directions. As the emission travels through the medium, new emissions are stimulated in a cascade. In lasers an essential component is a pair of mirrors

that flank the medium. The mirrors redirect emission back and forth through the medium for an efficient cascaded amplification continuously pumped into higher intensities. In inherently pulsed lasers at first the pumping accumulates excitation at a faster rate than relaxation occurs. Eventually the cascade of the stimulated emission driven by pumping and mirroring depopulates the majority of the excitation in concert, creating a burst or pulse of emission that constitutes the laser beam. Continuous wave (CW) lasers have during pumping a stable ground state population where the rate of relaxation is faster than the rate of excitation, ensuring a continuous supply of emission. Lasers capable of continuous irradiation can also be operated in a pulsing mode. The medium defines the laser output and may be a solid, gas, or liquid, as well as decides the type of pumping.

1.4.1 Lasers in tandem MS UVPD

Primarily two types of lasers are used in UVPD to fragment the peptide backbone [53]. Excimer lasers use a gas as the medium and electric discharge as the pumping mechanism. Excimer lasers are used for a pulsing UV output. The 193 nm output of the excimer laser applied in UVPD originates from ArF as the active medium. The inactive medium is Ar and F_2 in the ground state. Electrical discharge produces ArF which only exist in an excited state used for the UV output. Upon emission the heterodiatomic molecules dissociates to regenerate argon and molecular fluorine. A disadvantage of excimer lasers are their size, which could be one of the reasons why the 193 nm laser was not chosen by Thermo Scientific in the commercialized UVPD.

The Nd:YAG laser used for the commercialization of UVPD at 213 nm is significantly smaller and can be installed inside the cabinet of the Orbitrap Fusion Tribrid instruments. Nd:YAG is a solid-state medium of neodymium incorporated (or doped) in the crystal of yttrium aluminium garnet ($Y_3Al_5O_{12}$). Solid-state media are (with a few exceptions) pumped by external light sources in the wavelength region the medium absorbs. It is the transition metal in the transparent support that absorbs the external pumping and produces the irradiation. Nd:YAG has an output of 1064 nm which through pulse mode

operation and fifth harmonic generation is converted to the five-fold shortened wavelength of 213 nm applied for UVPD. In harmonic generation the photons are frequency-multiplied *n* times by the interaction between *n* initial photons from the pulsing laser. The initial *n* photons are merged to produce one combined new photon of *n* times higher energy and thereby *n* times shorter wavelength. By the selection of the appropriate laser output and harmonic generation, a variety of new wavelengths (also in the UV range) can be accessed.

1.4.2 UV femtosecond irradiation on biomolecules

An advantage of UV light is the interaction with biomolecules in living cells. Powerful pulses in the UV range produced by femtosecond lasers have been used to crosslink DNA-protein complexes *in vivo* [88-90]. These studies target the DNA-crosslinked proteins of interest with immunoprecipitation to reveal the bound DNA sequences, enabling mapping of the genomic sites that proteins bind to. The UV light-induced crosslinking by the short femtosecond pulsing are advantageous over the slower chemical crosslinking (like formaldehyde) when studying transient interactions. Pulsing lasers such as in the femtosecond timescale has been a major improvement over the continuous sources in increasing the yield of the DNA-protein crosslinking. The nucleic bases in DNA are UV chromophores capable of biphotonic reactivity that is efficient for inducing crosslinking [91]. The high intensity in laser pulses promote such multiphoton processes. Examples of crosslinking between amino acid residues in peptides and proteins induced directly by femtosecond pulsing have also been reported. In an *in vitro* peptide-peptide study, it was shown that the UV irradiation induced intermolecular crosslinks between two Trp or a Trp and a Tyr residue [92]. Another protein-protein study *in vivo* demonstrated it was possible with UV femtosecond pulsing to crosslink a dehydrogenase into dimerism [93]. The Petersen lab has reported the immobilization of proteins using femtosecond laser irradiation at 280 nm to induce crosslinking onto biosensor surfaces in a methodology coined Light Assisted Molecular Immobilization (LAMI) [94-96].

1.4.3 Femtosecond laser setup for 280 nm irradiation

In the Petersen lab in Aalborg University a customized setup with a solid-state Ti:sapphire femtosecond laser is installed. Ti:sapphire lasers have an unusual broad emission between ~700 nm to ~1100 nm that derives from a continuous access to vibrational energy levels that the excited electron can relax into before emission [97]. The method known as mode-locking is used to produce pulses in the order of femtosecond duration and is also applied to tune the wavelength of the output. Green light at 532 nm is pumped into the femtosecond laser tuned to produce an output at 840 nm, which can be frequency-tripled with a harmonic generator module to 280 nm (**Figure 4**). Mirrors ensure the travel of the laser beam between the femtosecond laser all the way to a cuvette sample holder. Samples of 3 mL can be irradiated in

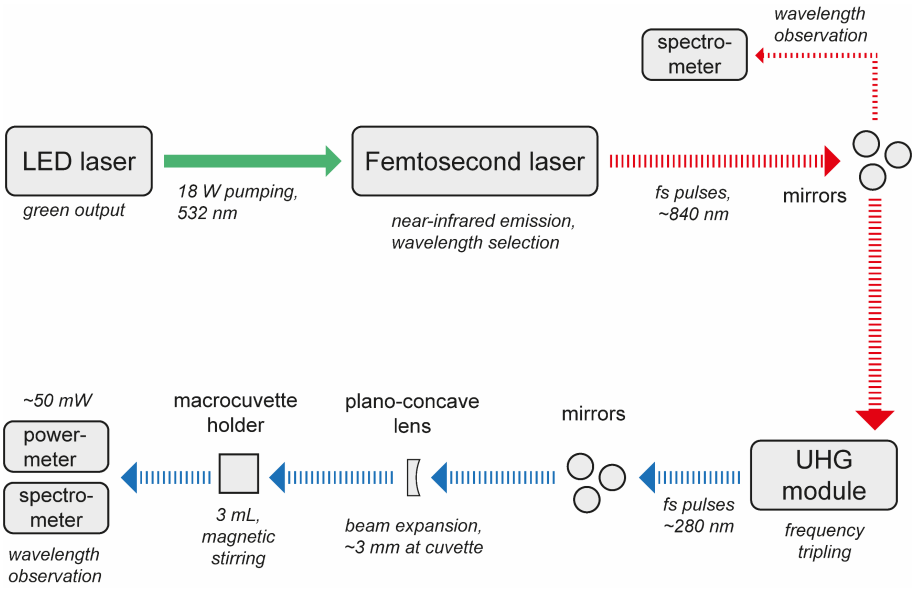

Figure 4. Simplified overview of the UV femtosecond laser setup in the Petersen lab, Aalborg University. The lasers and UHG module are all from Spectra Physics. A LED laser (Millennia eV) pumps the femtosecond laser (Tsunami XP). The pulses from the femtosecond laser are directed to frequency tripling in an ultrafast harmonic generation (UHG) and further directed to the sample in a macrocuvette.

macrocuvettes under magnetic stirring in the sample holder. Spectrometers measure the spectrum of the beam before and occasionally after the frequency tripling to validate the expected wavelengths. Between the last mirror and the sample holder a plano-concave lens is placed to expand the diameter of the laser beam to about 3 mm in diameter in the cuvette. The power was measured behind the sample holder in the absence of a cuvette. A great deal of energy is lost in the entire setup from the processes in the femtosecond laser, from the reflection by the many external mirrors, and from the frequency tripling. When the femtosecond laser is pumped with 18 W at 532 nm, a stable power level at 280 nm was usually 50 mW. Other parameters are a pulse duration of ~100 femtoseconds with a repetition rate of 80 million pulses pr. second (80 MHz).

New chapter next page

2 Research initiation

2.1 Motivation for the industrial PhD project

The Petersen lab (Aalborg University) has expertise in conducting UV photoreactions of proteins in solution with a powerful laser UV source and analysing the outcome with advanced spectroscopy. Among the UV light-induced modifications of interest were reduction of disulfide bonds. The Petersen lab sought for detailed knowledge of the covalent modifications in the individual UV photoproducts generated in the photonics lab. The department *MS Characterisation*, (Global Research Technologies, Novo Nordisk) are specialized in characterising peptides, proteins, and degradation products with LC-UV-MS. *MS Characterisation* was interested in developing new analytical methods, such as new ways of reducing disulfide bonds. Combining MS with powerful laser technology to drive UV photoreactions and characterise photoproducts of peptides/proteins in solution is an appealing opportunity that has only been explored to a limited extend. It is an important advantage for experimental procedures in industrial biopharmaceutic research that they are easy and fast, require minimal sample preparation, and that any experimental alteration fit existing pipelines. An interesting advantage of UV irradiation is that it is online-compatible with LC-MS, potentially allowing an efficient LC-laser-MS setup.

2.2 Objectives

The reported UV photoreduced products of disulfide proteins in solution have so far been perceived as degradation products suffering from UV photodamage. No previous studies have evaluated the analytical value of these in-solution UV photoproducts. The aims were divided in three parts:

1) The initial aim was to explore if and how UV light technologies could improve LC-MS analyses in disulfide bond mapping of biopharmaceuticals in simple procedures replacing chemical agents with light. We hypothesized that the intrinsic properties of Tyr/Trp residues upon UV excitation could be utilized as an electron source for the reduction of disulfide bonds in peptides/proteins. This aim is addressed in *Manuscript 2*.

2) The targeting of Tyr/Trp with UV light to initiate electron ejection may also activate other reaction pathways. The aim was therefore also to characterise major degradation products, study the putative electron-donated residues, and determine selectivity for photoreduction of disulfide bonds. This aim is addressed in *Manuscript 1, Manuscript 2,* and *Report 1*.

3) The first two aims cover in-solution studies, where the UV light irradiation is used as a preparative step before the LC-MS analysis. During the project an Orbitrap Fusion Lumos was upgraded with 213 nm UVPD. In the last part of the project the aim was also to test the analytical utilization of the UV laser and study the product ions generated from the gas-phase photoreactions. This aim is addressed in *Manuscript 2* and *Manuscript 3*.

As the project progressed the focus was shifted towards objectives 2 and 3.

2.3 UV source instrumentation and workflow

The Tyr/Trp-mediated photoreduction model was planned to be utilized in the wavelength region ~275-280 nm due to the local absorption maximum of these aromatic side chains. Two different types of UV light instrumentation in Petersen's photonics lab were identified as useful 280 nm light sources. A spectrofluorometer was used for CW irradiation with a xenon lamp coupled to a diffraction grating for wavelength selection. The advantages are an easy-to-use UV light source that is a complete commercial solution and does not require an advanced custom setup of photonic equipment. During irradiation the spectrofluorometer was used to measure real-time the degraded fluorescence of Tyr/Trp as the residues were degraded into products. The second UV light source was a pulsing femtosecond laser in a custom setup designed for output in the 280 nm region (**Figure 4**). We expected a significant power increase and acceleration of UV photoreactions compared to the CW xenon lamp in the spectrofluorometer. The UV irradiation was conducted offline to the LC-MS analysis. After the UV irradiation step in Petersen's lab, the samples were stored frozen and transported to *MS Characterisation*. The remaining workflow continued in the MS lab.

2.4 Strategy of the model molecules

The model of Tyr/Trp-mediated UV photoreduction and UV photoproducts have not previously been studied with LC-MS in the Petersen's lab. We selected a group of molecules varying in complexity to gradually study the model (**Figure 5**). The tripeptides Gly-Tyr-Gly (GYG) and Gly-Trp-Gly (GWG) were designed as the basic model peptides to characterise the products of Tyr and Trp after the putative electron ejection. Two biological peptides with a disulfide bond and a Tyr or Trp were selected as simple systems to study the hypothesized interaction between Tyr/Trp and the disulfide bond: arginine-vasopressin is a 9-residue peptide with one Tyr and one disulfide bond while somatostatin-14 is a 14-residue peptide with one Trp and one disulfide bond. Human insulin was used as the representative of the small protein with a more complex molecular system that included four Tyr residues,

two inter-chain disulfide bonds, and one intra-chain disulfide bond. The advantage of insulin for studying UV photoreduction is the separation of its two backbone polypeptides (A- and B-chain) after reduction of the inter-chain disulfide bonds, which is easily detected in LC-MS analysis.

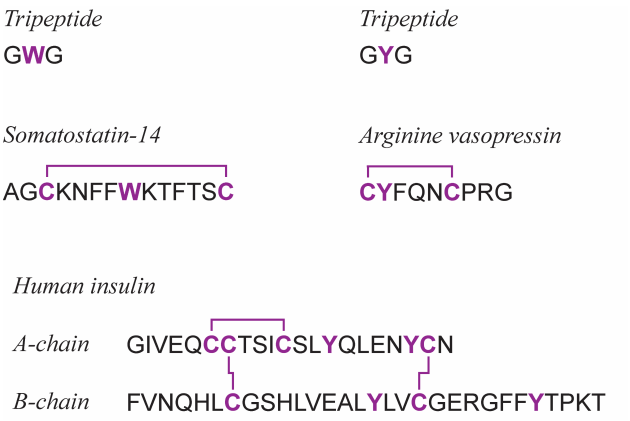

Figure 5. The molecules selected for the initial purpose of studying the model of Tyr/Trp-mediated UV photo-reduction of the disulfide bond.

3 Research outcomes

3.1 Overview of manuscripts & unpublished data

As the project progressed we assessed that the analytical value of UV light-induced reduction of disulfide bonds in proteins was limited and not worth the efforts of pursuing extensive optimization. We re-prioritized our aims towards the research of the combined utilization of UV laser technology and state-of-the-art MS. The common activities and focus throughout the manuscripts are therefore characterisation of UV laser-induced photoproducts using MS.

Manuscript 1 reports the advantages of using the femtosecond laser setup over the CW xenon lamp in accelerating the same 280 nm photoreactions arginine vasopressin and somatostatin-14. Utilizing the new powerful 280 nm source, photostability between the peptides were compared, and novel photomodifications as well as known were identified. After the femtosecond laser was established as a powerful source for driving UV photoreactions, we reported in ***Manuscript 2*** our attempts to induce and stabilize reduction of the disulfide bonds in vasopressin, somatostatin-14, and human insulin in the solution phase. Gas phase 213 nm UVPD in the MS instrument was also applied on the interchain disulfide bonds of insulin for a combined evaluation of the hypothesized Tyr/Trp-mediated UV photoreduction model. Among the major side-reactions to the UV light-induced reduction of insulin was covalent dimerization. ***Manuscript 3*** is a top-down MS study of the UV light-induced insulin dimer and a second insulin dimer from a different type of stress. The application of 213 nm UVPD for the characterisation of the insulin dimers were also studied. Diverse multistage approaches in the MS instrument were presented in the characterisation of the crosslinks and identification of their sites in the dimers.

Report 1 presents unpublished data of the tripeptides GYG and GWG. The same type of modification was observed between the molecules but in GWG it was intramolecular while with GYG it was intermolecular which lead to

dimerized products. The modification type was also observed for vasopressin, reported in *Manuscript 1* and *2*.

The manuscripts containing the detailed introductions, results, and discussions can be found in the Appendix. Summaries of the individual manuscripts are provided in the following chapters.

3.2 Summary of Manuscript 1

Motivation and comments. Initially we established that a simple workflow consisting of UV irradiation without any following sample preparation allowed for characterisation of photoproducts by LC-UV-MS. In the initial workflow the spectrofluorometer was exclusively used as the UV light source on the model molecules. Secondly, we wanted to know if the same UV photoproducts would arise from the CW xenon lamp (coupled to a diffraction grating in the spectrofluorometer) and the femtosecond pulsing laser. In a hypothetical comparison between CW and pulse irradiation at same power, the first would favour one-photon reactions while the second could to a larger probability activate multi-photon reactions. When the femtosecond laser setup replaced the spectrofluorometer for the 280 nm irradiation we observed the same photoproducts between the two UV light sources on all model molecules. This lead us to the opportunity to present the femtosecond laser technology as a more powerful alternative to the CW xenon lamp for activating the UV photochemistry of peptides. We also grasped the occasion to report identification of a series of UV photomodifications at pH 7.4. The following examples of UV photodamage can potentially induce major disruption of the function of peptides/proteins from altered side chains, misfolding, and oligomerization. The manuscript is entitled "*Characterization of Ultraviolet Photoreactions in Therapeutic Peptides by Femtosecond Laser Catalysis and Mass Spectrometry"*.

Results and discussion. The 280 nm photodegradation of the tripeptide GWG was compared between the excitation source of the spectrofluorometer and the

femtosecond laser setup. The half-life of the photodegradation of GWG was decreased 40-fold down to 34 s using the femtosecond laser setup. The laser setup was followingly demonstrated on the UV photochemistry study of somatostatin-14 and arginine vasopressin.

The 280 nm photodegradation of somatostatin-14 was exclusively localized to the Trp residue of which two types of major photomodifications were observed. The first was NFK, a common photo-oxidation product of Trp. The second modification was found in two photoproducts eluting separately in the chromatography with identical mass to somatostatin-14. Absorption spectroscopy coupled online to the chromatography revealed that the individual photoproduct of highest abundance contained modified chromophores due to two new local maxima of ~251 nm and ~284 nm. LC-MS^2 indicated a crosslink between Phe7 (which may account for the 251 nm) and Trp8 (which may account for the 284 nm). Such a Phe-Trp crosslink between aromatic side chains enables isomerism, why we also observed two photoproducts.

The 280 nm photodegradation of vasopressin was divided into a reaction pathway involving Tyr and pathways leaving the Tyr residue intact. After 30 s of laser irradiation, the Tyr fluorescence degraded ~3% while the total degradation on the molecule was 34%. LC-UV-MS analysis identified one Tyr-modified photoproduct, and a series of monomeric and dimeric photoproducts modified on the original disulfide bond. The Tyr photoproduct had a mass loss corresponding to NH_3 and a modified Tyr absorption profile due to a new local maximum of ~294 nm. The monomeric sulfur photoproducts included a monosulfide (thioether) and trisulfide species. The UV photoreaction of vasopressin was primarily dominated by dimeric species containing intermolecular disulfide bonding. Besides two purely scrambled dimeric products containing two intermolecular disulfide bonds, ten scrambled (and complicated) isomers were identified to harbour the sulfur modification dithiohemiacetal (reduced: R_1-CH(-SH)-S-R_2). The primary sulfur participated in intermolecular disulfide scrambling, forming dimers that had the additional modifications of intramolecular thioether crosslinks. How

ten dimeric isomers can be formed from dithiohemiacetal-vasopressin are studied and discussed in detail in the manuscript. The scrambled disulfide bonds were proposed to derive from UV photo-induced cleavage of the intrachain disulfide bond in monomeric vasopressin. Thiols were formed that due to the neutral pH subsequently reoxidized into scrambled intermolecular disulfide bonds causing the observed dimerization.

The differences in UV photoreactivity between somatostatin-14 and arginine vasopressin were striking and illustrate the challenges in predicting photostability solely on the primary structure. Even though the predicted extinction coefficient of Trp is 44-fold higher than for the disulfide bond, it took the femtosecond laser 4 min to degrade 33% of Trp in somatostatin-14 but only 30 s to degrade ~31% of the disulfide bond in vasopressin, which is an 8-fold difference. In contrast, the disulfide bond of somatostatin-14 was stable, further emphasizing that UV photoreactivity of amino acid polymers depends not only on sequence, but also on other characteristics which could be e.g. the folded structure.

3.3 Summary of Manuscript 2

Motivation and comments. The first attempts to UV photo-reduce disulfide bonds in the model molecules were conducted at pH 7.4. Our hypothesis that the observed scrambled disulfide were re-oxidized secondary products of UV photo-induced thiols lead us to irradiations conducted under acidic pH. It became evident that the side-reactions to the wanted UV photoreduction were dominating the photoreactions in our 280 nm targeting of Tyr in vasopressin and insulin. We realized that we were far away from a reliable and efficient disulfide bond mapping procedure using solely the intrinsic UV photochemistry of peptides/proteins – also because the disulfide bond of somatostatin-14 was completely stable. Characterisation of the successfully photoreduced products caught our attention as they did not meet our expectations. The Tyr residues in these in-solution reduced products were intact and lead us to following investigations of the mechanism behind UV light cleavage of the disulfide bonds that circumvented the initially

hypothesized model of Tyr/Trp-mediation. The manuscript is entitled "*Direct Ultraviolet Laser-Induced Reduction of Disulfide Bonds in Insulin and Vasopressin".*

Results and discussion. Disulfide bonds in vasopressin and human insulin were UV photoreducible but not in somatostatin-14. Re-oxidation of the UV photo-generated thiols were partially prevented at pH 3.0. In insulin we observed the cleavage of the interchain disulfide bonds due to free B-chain. The reduction of the disulfide bonds in reduced vasopressin and B-chain were supported by LC-MS^2 analysis, where analytical value was present in the significantly improved sequence coverage from fragmentation in the regions between the Cys residues. The quantity of degraded precursor of vasopressin or insulin that was converted to stable reduced product was ~3%. Accordingly, major side-products were also observed in our targeting of the aromatic residues with 280 nm light.

Peptide mapping of UV irradiated insulin at neutral pH identified three scrambled disulfide bonds, that additionally all had the same scrambled connectivity through a trisulfide variant. Common photo-oxidation modifications were also searched for to which we identified DOPA on Tyr14 of A-chain using a top-down MS^3 approach. However, the major reaction pathway of insulin was formation of HMWP observed as one continuous elution in the reverse-phase chromatography. The most abundant side-product to the UV photoreduction of vasopressin was the Tyr-modified species with a mass loss corresponding to NH_3 (previously reported in *Manuscript 1*). To ensure the disulfide bond was intact in this product, LC-MS^2 characterisation was performed. The NH_3 mass loss was within the Cys1-Tyr2 region, meaning the N-terminus must have been the leaving group in the photoreaction upon UV excitation of Tyr2.

We were not able to report any correlation between the requirement of Tyr/Trp and the observed reduction in the UV photoproducts characterised by LC-UV-MS^2. We suspected that the model of Tyr/Trp-mediation is not the exclusive mechanism in solution leading to UV photoreduction. We therefore

used a vasopressin-Tyr2Phe analogue and discovered it was also photoreducible under the same conditions. Cleavage of the disulfide bonds in vasopressin and insulin was also possible with 213 nm UVPD in the gas phase. Fragment species of separated A- and B-chain indicated that thiyl radicals were formed, which are known as intermediary products from the homolytic cleavage of the disulfide bond. Quantification of the reduced photoproducts in solution supported that the UV light-induced reduction was a one-photon reaction. Combining the information with the disulfide bond as significant UV chromophore, a new model was proposed to describe Tyr/Trp-independent UV photoreduction of disulfide bonds in solution. We proposed that when the disulfide bond directly absorbs one UV photon, the energy homolytically dissociates the disulfide group in the solution. Followingly, acidic pH promotes stabilization of the thiols while increased pH directs the equilibration towards re-oxidation that can recombine into non-native disulfide bonds in peptides/proteins.

3.4 Summary of Manuscript 3

Motivation and comments. In human insulin the primary reason for the observed low specificity of the interchain disulfide photoreduction (reported in *Manuscript 2*) was the dominating formation of HMWP. Conventional characterisation of crosslinks in insulin HMWP is challenging and time-consuming since it usually requires purification steps and peptide mapping of the singly isolated dimer variant. We took advantage of the opportunity to efficiently generate large quantities of insulin dimer with the femtosecond UV laser technology to conduct method development of MS-based characterisation of insulin dimers. At the same time we acquired characterisation of the UV light-induced dimer and was able to study 213 nm UVPD. The positive results motivated us to further explore top-down characterisation and UVPD on a second insulin dimer, induced by incubation of $FeSO_4$ as another type of stress. The manuscript is entitled "*Characterization of Insulin Dimers by Top-Down Mass Spectrometry"*.

Results and discussion. Denaturing size-exclusion chromatography showed that weeks of $FeSO_4$ incubation induced dimers while minutes of UV irradiation induced dimers as well as species at higher oligomeric states. The most abundant dimer from the two stress types were selected for two case studies. The intact mass of the Fe-induced dimer had the addition of a carbon (+12 Da) while the UV light-induced dimer suggested a modification corresponding to an addition of oxygen minus two hydrogen (+14 Da, +O-2H).

Identification of which two of the four chains in the dimer were crosslinked was confidently achieved with EThcD-MS^2 due to efficient cleavage of the interchain disulfide bonds. The monomers in the Fe-induced dimer were crosslinked between two B-chain and in the UV light-induced dimer the crosslink was between an A- and B-chain. Characterisation at the MS^2 level of the crosslinks in both dimers contained surprises that was not revealed by HCD but was with EThcD and 213 nm UVPD. The carbon crosslink in the Fe-induced dimer was indicated to crosslink to the aromatic side chain of Phe. The crosslink in the UV-light induced dimer contained an unusual O-S bond.

Crosslink site identification was possible for both dimers using MS^3 analysis of MS^2 fragments that were cleaved somewhere in the crosslink or the interchain disulfide bonds as well as both. In the Fe-induced dimer, the residual sites were between Phe1 of both B-chains. In the UV light-induced dimer, the addition of oxygen was localized to Tyr14 of A-chain and the involved sulfur was localized to both Cys residues of B-chain, meaning there was isomerism in the sulfur donation from B-chain to the O-S bond.

None of the fragmentation methods at the MS^2 level were able to provide crosslink identification in the UV light-induced dimer. However, for the Fe^{2+}-induced dimer, 213 nm UVPD at the MS^2 level provided significant advantage over the remaining activation methods with rich and extensive fragmentation close to and in the crosslink for confident identification of residual sites and sub-residual information. Among others, fragmentation of the C_α-C_β bond in Phe was observed. It is in the manuscript proposed and discussed in more detail that the success of 213 nm UVPD may be due to the direct crosslinking

to the chromophoric side chain of Phe1 as well as the remaining nearby chromophoric side chains that all function as significant collectors of the energetic 213 nm photons.

A shortcoming in our workflow was the lack of chromatography coupled to the MS instrument to potentially separate dimeric variants, which is a challenge on its own. However, the unlimited acquisition time to collect hundreds of scans were essential for acquiring confident spectra, which is not compatible with the time scale of an elution peak. Overall, we concluded that useful information can be extracted from insulin dimers with state-of-the-art MS in a top-down approach.

3.5 Summary of Report 1

Motivations and comments. The tripeptides GYG and GWG were in the beginning of the project studied to increase our understanding of the identity of the putative electron-donated Tyr/Trp in disulfide reduced photoproducts in the remaining model molecules. After having also characterised the photoreduced products in vasopressin and insulin, there appeared to be no direct correlation between the UV photoreactions of GYG/GWG and UV photoreduction of disulfide bonds. However, there were value in the comparisons between GYG and vasopressin as well as between GWG and somatostatin-14. Vasopressin has a Tyr residue at the second position just like the tripeptides. In somatostatin-14 the Trp residue is located away from the N-terminus and therefore different UV photo-characteristics were observed compared to GWG. The report is entitled "*The Cyclic Ultraviolet Photoproducts of the Tripeptides Gly-Tyr-Gly and Gly-Trp-Gly: Deamination and Aromatic Side Chain Crosslinking*".

Results and discussion. The 280 nm photoreaction of GWG lead to one major (93%) and one minor (7%) photoproduct. In both cases the modification corresponded to a total mass loss of NH_3. In such a simple molecule the N-terminus is an obvious candidate for the deamination, which was also supported by LC-MS^2. Online absorption spectroscopy measured modified

Trp profiles in both products, meaning that the indole side chains were crosslinked. The fragmentation also supported that it was the C_α atom in Gly1, previously bound to the N-terminus, which was crosslinked to the indole side chain. The mechanism of the deamination and side chain crosslinking is discussed in the report, which includes intra-molecular electron transfer from the aromatic side chain to the N-terminus. For GYG, deamination was also observed but here the crosslinking was inter-molecular. Seven dimers were observed, which all contained a total mass loss corresponding to two NH_3 moieties. The LC-MS^2 supported a dimeric structure where the phenol side chain of one monomer was crosslinked to the C_α of the other monomer.

The UV photoreaction of arginine vasopressin lead to one major Tyr product, also with the mass loss of one NH_3. Why the modification resembled GWG and not the dimerization of GYG could be due to the disulfide bond positioning the Tyr residue for an intramolecular photo-induced reaction with the N-terminus. The 280 nm half-life of GWG was 11-fold shorter than for somatostatin-14, indicating that having an aromatic residue positioned close to an N-terminus may be highly destabilizing under UV light exposure.

New chapter next page

4 Closing remarks

UV light technology is on its own without any support of chemicals unfit for reliable and efficient mapping procedures of disulfide bonds in proteins. The specificity of those disulfide bonds that we did succeed in reducing with the UV laser and detect with LC-UV-MS was too low and not promising for follow-up optimization studies. Scrambled disulfide bonds were observed, lowering the confidence in any mapped disulfide bond. Also, a range of diverse and unpredictable modifications can be introduced upon UV irradiation that increases complexity of data analysis – all specific to the protein of interest, which is not applicable in an organization requesting fast and reliable workflows.

The Tyr/Trp-mediated hypothesis have here been challenged as the only phenomenon in UV photo-induced reduction of disulfide bonds in solubilized proteins. The series of protein in-solutions studies throughout the ‘00s and ‘10s have advocated for the Tyr/Trp-mediated model or used it to rationalize observations. Discussions of mechanisms based on direct absorption of the photon by the disulfide bond have in comparison been limited. We hope to impact a more nuanced view in the in-solution UV photochemistry field on how disulfide bonds in peptides and proteins can become reduced with UV light so that researchers may conduct and rationalize their studies more successfully.

Throughout our UV irradiation experiments all the amino acid polymers yielded photoproducts. If one general predictability should be proposed, it is that upon sufficient UV light stress you should expect photoproducts that are detectable and characterizable with LC-UV-MS. Currently, UV light stability does not seem to be a major challenge for the biopharmaceutic industry with the current manufacturing, protective packaging, products, and costumers. It appears that there is awareness among researchers that heat-stable compounds could become correspondingly important as biopharmaceuticals are increasingly distributed to development countries with hot climates and limited access to cooling. If combined with a potential misadministration of the product by the user, product quality and patient safety could be at risk. In

this context, including light stability in the research and development of biotherapeutics could have an increased importance for patients from these sunny countries.

Not surprisingly the femtosecond technology was potent in accelerating the UV photoreactions. The equipment in the photonics lab was designed to support a variety of experimental setups. The performance of the laser was not optimized specifically to our experiments. Further significant potential remains untapped in the femtosecond laser technology for acceleration UV photoreactions of peptides and proteins. What did surprise us was the identical photoproducts between the CW source and the femtosecond-pulsing source, suggesting powerful pulsing sources may not be a general issue in UV photo-studies of peptides and proteins. The 213 nm laser for gas phase UVPD provided unusual deliveries in our work. We utilized UVPD to substantiate in-solution cleavage of the disulfide bond from direct photon absorption as well as fragmented a crosslinked UV chromophore in an insulin dimer for analytical value.

The coupling between gas phase laser-induced UVPD and MS has for years proven useful in the field of analytical peptide/protein chemistry, and also proven useful in our work. The reported utilization of UV lasers on in-solution peptides/proteins and MS is in the recent years scarce. In this thesis we additionally demonstrated that the pipeline of advanced UV laser technology and MS can be a powerful and successful combination in the research of UV photo-properties of peptides and proteins in solution.

5 Future perspectives

Ideas and thoughts that have crossed the mind are here shared on the conceptual level. They may be interesting to pursue if the solutions are achievable and provide significant impact in their respective fields.

Optimization of the laser setup. The utility of the femtosecond laser setup as a more powerful UV source than the xenon lamp-diffraction grating system is one advantage. Another feature of lasers we did not exploit to the fullest is the narrow width of the parallel beam, enabling irradiation of small targets. The sample volume of 3 mL used in the experiments is a large quantity compared to the 1 to 10 µL (and occasionally 20 µL) used for one LC-MS injection. In theory another acceleration by the factor of 40 can be deduced when decreasing the sample volume from 3 mL to 75 µL (under the assumption that the travel length of 1 cm for the laser beam through the sample is the same when decreasing the volume – however this is not realistic with 75 µL). Also, the photonics setup in the Petersen lab was built for diverse experimental opportunities and not optimized to the application in this thesis. For each redirection of the laser beam with a mirror about 10% of the intensity can be lost (Steffen Petersen, oral communication). About five of the mirrors can be avoided in an optimized construction.

UV light and lasers as a tool for rapidly generating HMWP at large quantities. The irradiation at 50 mW and 280 nm within minutes converted the majority of insulin to HMWP. Perhaps in other fields or applications such a generation of HMWP by a UV laser beam may have uses where other methods are used to induce formation of HMWP. E.g. in the testing of the separation power in size-exclusion columns. However, the formation of HMWP by UV light should be expected to differ from other stress types.

***In vivo* UV photomodifications studied by proteomics.** MS analysis of UV light-induced modifications of peptides and proteins have so far only been conducted *in vitro* of purified samples in buffers. Today LC-MS in proteomics

offers standardized workflows that are compatible with any stimuli that change the proteoform landscapes within the cells. Modifications could be searched for in epidermal cell lines exposed to a variety of UV light conditions varying in intensity, duration, and wavelengths. No one has so far conducted such studies to our knowledge. An opportunity also arises to study the cellular repair response induced by UV light in the case of damage on the DNA and protein level.

Implementation of high-powered pulsing lasers in photostability guidelines. The approved UV sources in the photostability guidelines by the authorities are all lamps [4]. For the five molecules, we always observed the same UV photoproducts between the continuous lamp and the pulsing laser setup. If it was further explored and established that pulsing sources do not introduce artificial modifications, the guidelines could be expanded to include pulsing lasers for rapid testing of photostability.

UV light-induced reduction with external chromophoric donor of electrons. What if it was possible to find or design a strong chromophoric compound that upon UV excitation readily releases electrons? The compound could then be co-solubilized with the protein and any progression of reduction can be controlled by the irradiation to facilitate a possible diverse production of partial reductions in proteins for disulfide mapping. Maybe such a putative ejection of electrons would be able to reduce buried disulfide bonds unreachable with conventional chemicals. To avoid excitation on the protein, such a light-triggered reductant should preferably absorb above 300 nm.

LC-MS online reduction of disulfide bonds with UV laser. In hydrogen-deuterium exchange MS, low temperature and pH (~2.5) are used to preserve the labelling of deuterium [98, 99]. Any sample preparation conducted to facilitate the MS analysis must be done rapidly to minimize the loss of labelling. One sample preparation is the reduction of disulfide bonds. Chemical reduction is normally applied but a common challenge since the

reduction is strongly inhibited by the low temperature and acidic pH [43, 45]. During the reducing step, labelling is lost with time, which forces a trade-off between reduction and preserving the labelling. A solution online to LC-MS saves the step of chemical reduction. A UV laser beam that cleaves the disulfide bonds could target the tubing between the column and MS instrument. Opposite to chemical reduction, we learned in *Manuscript 2* that UV light is directly compatible with acidic pH. The absorption of UV light is also not expected to be inhibited by any cold temperature if used during the chromatography. It may be so that the yield of disulfide reduction is significantly improved in the online coupling between chromatography, irradiation, and immediate MS analysis when compared to the results presented in *Manuscript 2,* in which offline irradiation could allow time for re-formation of the disulfide bond and formation of secondary products. The organic solvents in the reversed-phase chromatography are commonly methanol or acetonitrile, which could facilitate a significant improvement in the yield of UV photo-cleaved disulfide bonds (as was demonstrated by the Yu Xia lab [86, 87]) in the deuterium-containing peptides.

UVPD at ~280 nm targeting Tyr and Trp. It could be interesting to study if any selective fragmentation of the backbone in proximity to Tyr and Trp can be induced if only these two residues are excited. Selective fragmentation at Tyr and Trp would provide predictable and limited fragmentation similar to an in-solution protease digestion. A mechanism to induce backbone fragmentation could be transfer of excitation from Tyr and Trp to the peptide bond. However this may be impossible to accomplish as the 280 nm excitation of the aromatic side chain of Tyr or Trp should not match any electronic transition in the peptide bond as here excitation occurs at lower wavelengths. Another mechanism may be through radicalization from the fragmentation of the C_α-C_β bond.

Characterisation of aromatically-crosslinked products with 213 nm UVPD. Throughout the project we identified multiple photoproducts that

contained crosslinks in aromatic side chains. The Phe-Trp crosslink of somatostatin-14, and the deamination (minus NH_3) products of vasopressin, GYG, and GWG could be re-analysed with 213 nm UVPD to search for new unique fragments that contribute to the characterisation of the modifications.

Top-down MS of HMWP induced by aromatic formulations with 213 nm UVPD. Aromatic compounds such as phenol may be used in formulation of biotherapeutics to improve chemical stability towards oxidation. Phenol is also used as an antibacterial agent for multiple injection cartridges. However, formulations may also participate in the formation of HMWP. If the crosslink from the formulation is suspected to be aromatic, top-down fragmentation of the dimer with 213 UVPD could have higher specificity for the crosslink than the peptide bond, providing analytical information about the crosslink.

An outlook that we evaluate is promising, and with a void in discoveries, is UVPD of crosslinked chromophores such as the intrinsic aromatic side chains or extrinsically conjugated compounds, covered by the last two perspectives.

6 References

1. Gammelgaard, S.K., et al., *Characterization of Ultraviolet Photoreactions in Therapeutic Peptides by Femtosecond Laser Catalysis and Mass Spectrometry.* ACS Omega, 2019. **4**(11): p. 14517-14525.
2. Gammelgaard, S.K., et al., *Direct Ultraviolet Laser-Induced Reduction of Disulfide Bonds in Insulin and Vasopressin.* ACS Omega, 2020. **5**(14): p. 7962-7968.
3. Bessa, J., et al., *The immunogenicity of antibody aggregates in a novel transgenic mouse model.* Pharm Res, 2015. **32**(7): p. 2344-59.
4. *ICH Harmonised Tripartite Guideline. Stability Testing: Photostability Testing of New Drug Substances and Products Q1B.* 1996; Available from: https://database.ich.org/sites/default/files/Q1B%20Guideline.pdf.
5. Goldfarb, A.R., L.J. Saidel, and E. Mosovich, *The ultraviolet absorption spectra of proteins.* J Biol Chem, 1951. **193**(1): p. 397-404.
6. Beaven, G.H. and E.R. Holiday, *Ultraviolet Absorption Spectra of Proteins and Amino Acids*, in *Advances in Protein Chemistry*, M.L. Anson, K. Bailey, and J.T. Edsall, Editors. 1952, Academic Press. p. 319-386.
7. Pace, C.N., et al., *How to measure and predict the molar absorption coefficient of a protein.* Protein Sci, 1995. **4**(11): p. 2411-23.
8. Grimsley, G.R. and C.N. Pace, *Spectrophotometric Determination of Protein Concentration.* Current Protocols in Protein Science, 2003. **33**(1): p. 3.1.1-3.1.9.
9. Kuipers, B.J. and H. Gruppen, *Prediction of molar extinction coefficients of proteins and peptides using UV absorption of the constituent amino acids at 214 nm to enable quantitative reverse phase high-performance liquid chromatography-mass spectrometry analysis.* J Agric Food Chem, 2007. **55**(14): p. 5445-51.
10. Anthis, N.J. and G.M. Clore, *Sequence-specific determination of protein and peptide concentrations by absorbance at 205 nm.* Protein science : a publication of the Protein Society, 2013. **22**(6): p. 851-858.
11. Kerwin, B.A. and R.L. Remmele, Jr., *Protect from light: photodegradation and protein biologics.* J Pharm Sci, 2007. **96**(6): p. 1468-79.

12. Pattison, D.I., A.S. Rahmanto, and M.J. Davies, *Photo-oxidation of proteins.* Photochem Photobiol Sci, 2012. **11**(1): p. 38-53.
13. Parker, N.R., et al., *Protein-bound kynurenine is a photosensitizer of oxidative damage.* Free Radical Biology and Medicine, 2004. **37**(9): p. 1479-1489.
14. Lam, M.A., et al., *Nitric Oxide and Nitroxides Can Act as Efficient Scavengers of Protein-Derived Free Radicals.* Chemical Research in Toxicology, 2008. **21**(11): p. 2111-2119.
15. Gracanin, M., et al., *Singlet-oxygen-mediated amino acid and protein oxidation: formation of tryptophan peroxides and decomposition products.* Free Radic Biol Med, 2009. **47**(1): p. 92-102.
16. Ronsein, G.E., et al., *Characterization of O2 (1Δg)-Derived Oxidation Products of Tryptophan: A Combination of Tandem Mass Spectrometry Analyses and Isotopic Labeling Studies.* Journal of the American Society for Mass Spectrometry, 2009. **20**(2): p. 188-197.
17. Grosvenor, A.J., J.D. Morton, and J.M. Dyer, *Profiling of residue-level photo-oxidative damage in peptides.* Amino Acids, 2010. **39**(1): p. 285-96.
18. Grosvenor, A.J., J.D. Morton, and J.M. Dyer, *Isobaric Labeling Approach to the Tracking and Relative Quantitation of Peptide Damage at the Primary Structural Level.* Journal of Agricultural and Food Chemistry, 2010. **58**(24): p. 12672-12677.
19. Qi, P., et al., *Characterization of the photodegradation of a human IgG1 monoclonal antibody formulated as a high-concentration liquid dosage form.* Journal of Pharmaceutical Sciences, 2009. **98**(9): p. 3117-3130.
20. Li, Y., et al., *Characterization of the Degradation Products of a Color-Changed Monoclonal Antibody: Tryptophan-Derived Chromophores.* Analytical Chemistry, 2014. **86**(14): p. 6850-6857.
21. Sreedhara, A., et al., *Effect of ambient light on IgG1 monoclonal antibodies during drug product processing and development.* European Journal of Pharmaceutics and Biopharmaceutics, 2016. **100**: p. 38-46.
22. Wecksler, A.T., et al., *Photodisruption of the Structurally Conserved Cys-Cys-Trp Triads Leads to Reduction-Resistant Scrambled Intrachain Disulfides in an IgG1 Monoclonal Antibody.* Mol Pharm, 2018. **15**(4): p. 1598-1606.
23. Schweikart, F. and G. Hulthe, *HPLC-UV-MS Analysis: A Source for Severe Oxidation Artifacts.* Anal Chem, 2019. **91**(3): p. 1748-1751.

24. Lehrer, S.S. and G.D. Fasman, *Ultraviolet Irradiation Effects in Poly-L-tyrosine and Model Compounds. Identification of Bityrosine as a Photoproduct*.* Biochemistry, 1967. **6**(3): p. 757-767.
25. Malencik, D.A. and S.R. Anderson, *Dityrosine formation in calmodulin.* Biochemistry, 1987. **26**(3): p. 695-704.
26. Correia, M., et al., *UV-light exposure of insulin: pharmaceutical implications upon covalent insulin dityrosine dimerization and disulphide bond photolysis.* PLoS One, 2012. **7**(12): p. e50733.
27. Zhao, Z., et al., *Generation of Aggregates of alpha-Lactalbumin by UV-B Light Exposure.* J Agric Food Chem, 2020. **68**(24): p. 6701-6714.
28. Haywood, J., et al., *Light-Induced Conversion of Trp to Gly and Gly Hydroperoxide in IgG1.* Molecular Pharmaceutics, 2013. **10**(3): p. 1146-1150.
29. Mozziconacci, O. and C. Schoneich, *Effect of conformation on the photodegradation of Trp- and cystine-containing cyclic peptides: octreotide and somatostatin.* Mol Pharm, 2014. **11**(10): p. 3537-46.
30. Kang, H., T.J. Tolbert, and C. Schoneich, *Photo-induced tyrosine side chain fragmentation in IgG4-Fc: mechanisms and solvent isotope effects.* Mol Pharm, 2018.
31. Schöneich, C., *Photo-Degradation of Therapeutic Proteins: Mechanistic Aspects.* Pharm Res, 2020. **37**(3): p. 45.
32. Tabarin, T., et al., *Specific photodissociation of peptides with multi-stage mass spectrometry.* Rapid Commun Mass Spectrom, 2005. **19**(20): p. 2883-92.
33. Antoine, R., et al., *Photo-induced formation of radical anion peptides. Electron photodetachment dissociation experiments.* Rapid Commun Mass Spectrom, 2007. **21**(2): p. 265-8.
34. Lepere, V., et al., *Characterization of neutral fragments issued from the photodissociation of protonated tryptophane.* Phys Chem Chem Phys, 2007. **9**(39): p. 5330-4.
35. Schoneich, C., *Thiyl Radical Reactions in the Chemical Degradation of Pharmaceutical Proteins.* Molecules, 2019. **24**(23).
36. Mozziconacci, O., B.A. Kerwin, and C. Schoneich, *Photolysis of an intrachain peptide disulfide bond: primary and secondary processes, formation of H2S, and hydrogen transfer reactions.* J Phys Chem B, 2010. **114**(10): p. 3668-88.
37. Mozziconacci, O., B.A. Kerwin, and C. Schoneich, *Exposure of a monoclonal antibody, IgG1, to UV-light leads to protein*

dithiohemiacetal and thioether cross-links: a role for thiyl radicals? Chem Res Toxicol, 2010. **23**(8): p. 1310-2.
38. Mozziconacci, O. and C. Schoneich, *Photodegradation of oxytocin and thermal stability of photoproducts.* J Pharm Sci, 2012. **101**(9): p. 3331-46.
39. Steinmann, D., et al., *Photodegradation Pathways of Protein Disulfides: Human Growth Hormone.* Pharm Res, 2017. **34**(12): p. 2756-2778.
40. Wu, L.-Z., et al., *Photoexcitation of tryptophan groups induced reduction of disulfide bonds in hen egg white lysozyme.* Journal of Molecular Structure, 2008. **882**(1): p. 101-106.
41. Xie, J., et al., *Mechanistic insight of photo-induced aggregation of chicken egg white lysozyme: the interplay between hydrophobic interactions and formation of intermolecular disulfide bonds.* Proteins, 2011. **79**(8): p. 2505-16.
42. Lakbub, J.C., J.T. Shipman, and H. Desaire, *Recent mass spectrometry-based techniques and considerations for disulfide bond characterization in proteins.* Anal Bioanal Chem, 2018. **410**(10): p. 2467-2484.
43. Cline, D.J., et al., *New Water-Soluble Phosphines as Reductants of Peptide and Protein Disulfide Bonds: Reactivity and Membrane Permeability.* Biochemistry, 2004. **43**(48): p. 15195-15203.
44. Zhang, Y., H.D. Dewald, and H. Chen, *Online mass spectrometric analysis of proteins/peptides following electrolytic cleavage of disulfide bonds.* J Proteome Res, 2011. **10**(3): p. 1293-304.
45. Trabjerg, E., et al., *Conformational analysis of large and highly disulfide-stabilized proteins by integrating online electrochemical reduction into an optimized H/D exchange mass spectrometry workflow.* Anal Chem, 2015. **87**(17): p. 8880-8.
46. Cramer, C.N., et al., *Disulfide Linkage Characterization of Disulfide Bond-Containing Proteins and Peptides by Reducing Electrochemistry and Mass Spectrometry.* Anal Chem, 2016. **88**(3): p. 1585-92.
47. Cramer, C.N., et al., *Complete Mapping of Complex Disulfide Patterns with Closely-Spaced Cysteines by In-Source Reduction and Data-Dependent Mass Spectrometry.* Anal Chem, 2017. **89**(11): p. 5949-5957.
48. Cramer, C.N., et al., *Generic Workflow for Mapping of Complex Disulfide Bonds Using In-Source Reduction and Extracted Ion*

Chromatograms from Data-Dependent Mass Spectrometry. Anal Chem, 2018. **90**(13): p. 8202-8210.
49. Greaves, J. and J. Roboz, *Mass Spectrometry for the Novice*. 2013: CRC Press LLC.
50. de Hoffmann, E. and V. Stroobant, *Mass Spectrometry: Principles and Applications (third edition)*. 2013: Wiley.
51. Roepstorff, P. and J. Fohlman, *Proposal for a common nomenclature for sequence ions in mass spectra of peptides.* Biomedical mass spectrometry, 1984. **11**(11): p. 601.
52. Brodbelt, J.S., *Photodissociation mass spectrometry: new tools for characterization of biological molecules.* Chem Soc Rev, 2014. **43**(8): p. 2757-83.
53. Brodbelt, J.S., L.J. Morrison, and I. Santos, *Ultraviolet Photodissociation Mass Spectrometry for Analysis of Biological Molecules.* Chem Rev, 2020. **120**(7): p. 3328-3380.
54. Swaney, D.L., et al., *Supplemental Activation Method for High-Efficiency Electron-Transfer Dissociation of Doubly Protonated Peptide Precursors.* Analytical Chemistry, 2007. **79**(2): p. 477-485.
55. Frese, C.K., et al., *Toward Full Peptide Sequence Coverage by Dual Fragmentation Combining Electron-Transfer and Higher-Energy Collision Dissociation Tandem Mass Spectrometry.* Analytical Chemistry, 2012. **84**(22): p. 9668-9673.
56. R, R.J., *The Mechanism Behind Top-Down UVPD Experiments: Making Sense of Apparent Contradictions.* J Am Soc Mass Spectrom, 2017. **28**(9): p. 1823-1826.
57. Kolbowski, L., A. Belsom, and J. Rappsilber, *Ultraviolet Photodissociation of Tryptic Peptide Backbones at 213 nm.* Journal of the American Society for Mass Spectrometry, 2020. **31**(6): p. 1282-1290.
58. Scheffler, K., R. Viner, and E. Damoc, *High resolution top-down experimental strategies on the Orbitrap platform.* J Proteomics, 2018. **175**: p. 42-55.
59. Catherman, A.D., O.S. Skinner, and N.L. Kelleher, *Top Down proteomics: Facts and perspectives.* Biochemical and Biophysical Research Communications, 2014. **445**(4): p. 683-693.
60. Fellers, R.T., et al., *ProSight Lite: graphical software to analyze top-down mass spectrometry data.* Proteomics, 2015. **15**(7): p. 1235-8.
61. Wu, S.L., et al., *Mass spectrometric determination of disulfide linkages in recombinant therapeutic proteins using online LC-MS*

with electron-transfer dissociation. Anal Chem, 2009. **81**(1): p. 112-22.
62. Wu, S.L., et al., *Identification of the unpaired cysteine status and complete mapping of the 17 disulfides of recombinant tissue plasminogen activator using LC-MS with electron transfer dissociation/collision induced dissociation.* Anal Chem, 2010. **82**(12): p. 5296-303.
63. Wang, Y., et al., *Characterization and comparison of disulfide linkages and scrambling patterns in therapeutic monoclonal antibodies: using LC-MS with electron transfer dissociation.* Anal Chem, 2011. **83**(8): p. 3133-40.
64. Cole, S.R., et al., *Electron transfer dissociation (ETD) of peptides containing intrachain disulfide bonds.* J Am Soc Mass Spectrom, 2012. **23**(2): p. 310-20.
65. Clark, D.F., E.P. Go, and H. Desaire, *Simple approach to assign disulfide connectivity using extracted ion chromatograms of electron transfer dissociation spectra.* Analytical chemistry, 2013. **85**(2): p. 1192-1199.
66. Wen, D., et al., *Determination of the Disulfide Structure of Murine Meteorin, a Neurotrophic Factor, by LC–MS and Electron Transfer Dissociation-High-Energy Collisional Dissociation Analysis of Proteolytic Fragments.* Analytical Chemistry, 2017. **89**(7): p. 4021-4030.
67. Quick, M.M., et al., *Characterization of Disulfide Linkages in Proteins by 193 nm Ultraviolet Photodissociation (UVPD) Mass Spectrometry.* Anal Chem, 2018. **90**(14): p. 8523-8530.
68. Fornelli, L., et al., *Accurate Sequence Analysis of a Monoclonal Antibody by Top-Down and Middle-Down Orbitrap Mass Spectrometry Applying Multiple Ion Activation Techniques.* Anal Chem, 2018. **90**(14): p. 8421-8429.
69. Kruppa, G.H., J. Schoeniger, and M.M. Young, *A top down approach to protein structural studies using chemical cross-linking and Fourier transform mass spectrometry.* Rapid Commun Mass Spectrom, 2003. **17**(2): p. 155-62.
70. Webb, I.K., et al., *Gas-phase intramolecular protein crosslinking via ion/ion reactions: ubiquitin and a homobifunctional sulfo-NHS ester.* J Am Soc Mass Spectrom, 2013. **24**(5): p. 733-43.
71. Cammarata, M.B. and J.S. Brodbelt, *Characterization of Intra- and Intermolecular Protein Crosslinking by Top Down Ultraviolet*

Photodissociation Mass Spectrometry. ChemistrySelect, 2016. **1**(3): p. 590-593.
72. Fung, Y.M., et al., *Facile disulfide bond cleavage in gaseous peptide and protein cations by ultraviolet photodissociation at 157 nm.* Angew Chem Int Ed Engl, 2005. **44**(39): p. 6399-403.
73. Agarwal, A., J.K. Diedrich, and R.R. Julian, *Direct elucidation of disulfide bond partners using ultraviolet photodissociation mass spectrometry.* Anal Chem, 2011. **83**(17): p. 6455-8.
74. Soorkia, S., et al., *UV Photofragmentation Dynamics of Protonated Cystine: Disulfide Bond Rupture.* J Phys Chem Lett, 2014. **5**(7): p. 1110-6.
75. Prompers, J.J., C.W. Hilbers, and H.A. Pepermans, *Tryptophan mediated photoreduction of disulfide bond causes unusual fluorescence behaviour of Fusarium solani pisi cutinase.* FEBS Lett, 1999. **456**(3): p. 409-16.
76. Vanhooren, A., et al., *Photoexcitation of tryptophan groups induces reduction of two disulfide bonds in goat alpha-lactalbumin.* Biochemistry, 2002. **41**(36): p. 11035-43.
77. Vanhooren, A., et al., *Selectivity of tryptophan residues in mediating photolysis of disulfide bridges in goat alpha-lactalbumin.* Biochemistry, 2006. **45**(7): p. 2085-93.
78. Permyakov, E.A., et al., *Ultraviolet illumination-induced reduction of alpha-lactalbumin disulfide bridges.* Proteins, 2003. **51**(4): p. 498-503.
79. Neves-Petersen, M.T., et al., *High probability of disrupting a disulphide bridge mediated by an endogenous excited tryptophan residue.* Protein Sci, 2002. **11**(3): p. 588-600.
80. Neves-Petersen, M.T., et al., *Flash photolysis of cutinase: identification and decay kinetics of transient intermediates formed upon UV excitation of aromatic residues.* Biophys J, 2009. **97**(1): p. 211-26.
81. Mozziconacci, O., et al., *Reversible intramolecular hydrogen transfer between protein cysteine thiyl radicals and alpha C-H bonds in insulin: control of selectivity by secondary structure.* J Phys Chem B, 2008. **112**(49): p. 15921-32.
82. Abaskharon, R.M. and F. Gai, *Direct measurement of the tryptophan-mediated photocleavage kinetics of a protein disulfide bond.* Phys Chem Chem Phys, 2016. **18**(14): p. 9602-7.

83. Dose, K., *THE PHOTOLYSIS OF FREE CYSTINE IN THE PRESENCE OF AROMATIC AMINO ACIDS.* Photochemistry and Photobiology, 1968. **8**(4): p. 331-335.
84. Creed, D., *THE PHOTOPHYSICS AND PHOTOCHEMISTRY OF THE NEAR-UV ABSORBING AMINO ACIDS–II. TYROSINE AND ITS SIMPLE DERIVATIVES.* Photochemistry and Photobiology, 1984. **39**(4): p. 563-575.
85. Creed, D., *THE PHOTOPHYSICS AND PHOTOCHEMISTRY OF THE NEAR-UV ABSORBING AMINO ACIDS-III. CYSTINE AND ITS SIMPLE DERIVATIVES.* Photochemistry and Photobiology, 1984. **39**(4): p. 577-583.
86. Durand, K.L., et al., *Assigning Peptide Disulfide Linkage Pattern Among Regio-Isomers via Methoxy Addition to Disulfide and Tandem Mass Spectrometry.* J Am Soc Mass Spectrom, 2017. **28**(6): p. 1099-1108.
87. Adhikari, S., X. Yang, and Y. Xia, *Acetone/Isopropanol Photoinitiating System Enables Tunable Disulfide Reduction and Disulfide Mapping via Tandem Mass Spectrometry.* Anal Chem, 2018. **90**(21): p. 13036-13043.
88. Lejnine, S., et al., *Crosslinking of proteins to DNA in human nuclei using a 60 femtosecond 266 nm laser.* Nucleic Acids Res, 1999. **27**(18): p. 3676-84.
89. Zhang, L., et al., *Detecting DNA-binding of proteins in vivo by UV-crosslinking and immunoprecipitation.* Biochemical and Biophysical Research Communications, 2004. **322**(3): p. 705-711.
90. Altucci, C., et al., *Nonlinear protein - nucleic acid crosslinking induced by femtosecond UV laser pulses in living cells.* Laser Physics Letters, 2012. **9**(3): p. 234-239.
91. Meisenheimer, K.M. and T.H. Koch, *Photocross-linking of nucleic acids to associated proteins.* Crit Rev Biochem Mol Biol, 1997. **32**(2): p. 101-40.
92. Leo, G., et al., *Ultraviolet laser-induced cross-linking in peptides.* Rapid Commun Mass Spectrom, 2013. **27**(14): p. 1660-8.
93. Itri, F., et al., *Femtosecond UV-laser pulses to unveil protein-protein interactions in living cells.* Cell Mol Life Sci, 2016. **73**(3): p. 637-48.
94. Neves-Petersen, M.T., et al., *Photonic activation of disulfide bridges achieves oriented protein immobilization on biosensor surfaces.* Protein Sci, 2006. **15**(2): p. 343-51.
95. Gonçalves, O., et al. *Novel microfabrication stage allowing for one-photon and multi-photon light assisted molecular immobilization*

and for multi-photon microscope. in *SPIE BiOS*. 2017. Bellingham, WA: SPIE, The International Society for Optical Engineering.
96. Odete, G., et al. *Photonic immobilization techniques used for the detection of cardiovascular disease biomarkers*. in *Proc.SPIE*. 2018.
97. Andrews, D.L., *Lasers in Chemistry (third edition)*. 1997: Springer Berlin Heidelberg.
98. Zhang, Z. and D.L. Smith, *Determination of amide hydrogen exchange by mass spectrometry: a new tool for protein structure elucidation.* Protein science : a publication of the Protein Society, 1993. **2**(4): p. 522-531.
99. Walters, B.T., et al., *Minimizing back exchange in the hydrogen exchange-mass spectrometry experiment.* Journal of the American Society for Mass Spectrometry, 2012. **23**(12): p. 2132-2139.

7 Appendix

7.1 Manuscript 1

Characterization of Ultraviolet Photoreactions in Therapeutic Peptides by Femtosecond Laser Catalysis and Mass Spectrometry

Simon K. Gammelgaard, Steffen B. Petersen, Kim F. Haselmann, and Peter Kresten Nielsen
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7.2 Manuscript 2

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7.3 Manuscript 3

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7.4 Report 1

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